Effects of Enamel Matrix Derivative components on PDL cell differentiation

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Doctor of Philosophy

by

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DECLARATION

I, Harsh Devangbhai Amin confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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(Harsh Amin)

ABSTRACT

Previous studies have reported that the adult periodontal ligament (PDL) may contain progenitor/stem cells that function as precursors for periodontal tissue regeneration, although the ability of this population to differentiate into the multiple lineages present in the PDL is not yet certain. In addition, although Enamel Matrix Derivative (EMD; Emdogain®), derived from the enamel matrix of developing teeth) has been used extensively to help re-build new periodontal tissue, its effect on bone regeneration remains inconclusive and its effect on PDL blood vessels and nerve cell development not yet known, because it comprises a heterogeneous mixture of proteins. EMD has recently been separated into two main fractions: Fraction C, containing proteins < 6 kDa (mainly the tyrosine-rich amelogenin peptide TRAP); and Fraction A, containing proteins > 6 KDa (including the full-length amelogenin, sheathlins and a leucine-rich amelogenin peptide LRAP). The present study examined the effects of EMD Fractions on multi-lineage differentiation pathays of PDL cells *in vitro*. The results of the present study have shown that that Fraction C and Fraction A differentially regulate multi-lineage specification of PDL cells. Thus, Fraction C was found to up-regulate chondrogenic, vasculogenic, angiogenic, neurogenic and gliogenic genes and 'terminal' differentiation, whereas Fraction A was found to stimulate osteogenic genes and terminal osteogenic differentiation *in vitro*; both fractions suppressed adipogenesis. Moreover, the TRAP and LRAP peptides of Fraction C and Fraction A, respectively, were found to be at least partly responsible for the differential activities of these two fractions. In addition, at least some components in these EMD Fractions bound to and were internalized into PDL cells, most probably by receptor-mediated endocytosis. These findings thus demonstrate that the PDL contains cells with multi-lineage differentiation potential and that the components of EMD have differential effects on the diverse activities on the heterogeneous cells present in the PDL.

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AB	Alveolar bone
AF	Access Flap surgery
AgP	Aggressive Periodontitis
ALP	Alkaline phosphatase
AM	Adipogenic medium
Ang	Angiopoietin
AR	Arteries
BDNF	Brain-derived growth factor
bFGF	Basic-fibroblast growth factor
BMP	Bone morphogenetic protein
BMSC	Bone marrow derived mesenchymal stem cells
BSP	Bone sialoprotein
BV	Blood vessels
C	Allantoic capillaries
c-AMP	Cyclic adenosine 3' 5'- monophosphate
C/EBP	CCAAT/enhancer binding protein
C7	Clone 7
CAM	Chorio-allantoic membrane
CD	Cluster of differentiation
cDNA	Complimentary DNA
CM	Chondrogenic medium
CNS	Central nervous system
CP	Chronic Periodontitis
CREB	c-AMP response element binding protein
DEX	Dexamethasone
DFDBA	Demineralised freeze-dried bone allograft
DM	Differentiation medium

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EBM-2	Endothelial basal medium-2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEA-1	Early endosome antigen-1
EGF	Epidermal growth factor
EM	Electron microscopy
EMD	Enamel matrix derivative
EMP	Enamel matrix proteins
FCM	Flow cytometry
FCS	Foetal calf serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FSC	Forward scatter
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Glial differentiation medium
GF	Growth factors
GFAP	Glial fibril acidic protein
GM	Growth medium
GPI	Glycophosphatidylinositol
GTR	Guided Tissue Regeneration
HMVEC	Human microvascular endothelial cells
HRP	Horse red peroxides
HUVEC	Human umbilical vein endothelial cells
IBMX	3-isobutyl-1-methylxanthine

IGF-1	Insulin-like growth factor-1
LAMP-1	Lysosome-associated membrane protein-2
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LRAP	Leucine-rich amelogenin peptide
MAP-2	Microtubule-associated protein-2
MM	Myogenic medium
MMLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MOBP	Myelinated oligodendrocyte basic protein
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
NF-L	Neurofilament-light chain
NGF	Nerve growth factor
NGS	Normal goat serum
NM	Neural medium
NT-3	Neurotrophine-3
OC	Osteocalcin
Oct	Octamer-binding transcription factor
OM	Osteogenic medium
OP	Osteopontin
p-Smad	Phosphorylated Smad
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factors
PDL	Periodontal ligament
PE-CAM	Platelet/Endothelial cell adhesion molecule
PG	Proteoglycans
PGA	Propylene glycol ester of alginate
PGP9.5	Protein gene product 9.5

PNS	Peripheral nervous system
PPARγ-2	Peroxisome proliferator-activated receptor γ-2
Q-PCR	Quantitative polymerase chain reaction
RT	Room temperature
RT-PCR	Reverse-transcription polymerase chain reaction
Runx2	Runt-related transcription factor
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SE	Standard error
Sox	Sex determining region Y
SSC	Side scatter
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor β
TGN	Trans-Golgi network
TRAP	Tyrosine rich amelogenin peptides
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor
w/v	Weight per volume

I would like to dedicate this thesis to my parents and my late grand parents

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PUBLICATIONS

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PATENT

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PRESENTATION

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Chapter 1

1.1. Periodontal anatomy and function

The periodontium, a specialized tissue compartment responsible for tooth support, comprises cementum, periodontal ligament, alveolar bone and gingival tissue. Each of the periodontium-associated tissue has specialized anatomical structures and physiological relevance.

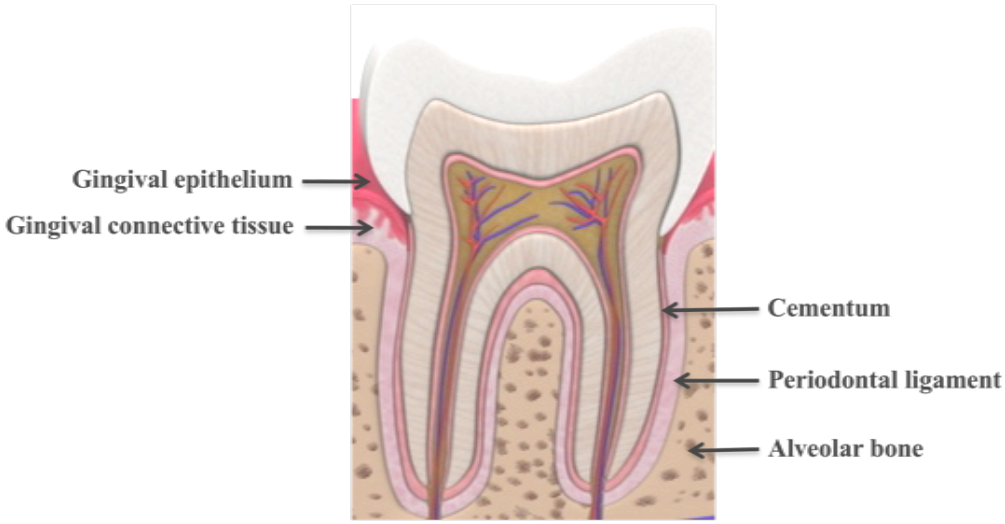


Figure 1.1 The structure of the healthy periodontium, comprising the gingival epithelium, gingival connective tissue, alveolar bone, cementum and periodontal ligament (PDL). (photograph taken from www.studiodentaire.com)

1.1.1. Cementum

Cementum, a mineralized avascular connective tissue that binds firmly to dentin and covers the entire root surface (Lindhe and Karring, 2009; Nanci et al., 2006), is believed to be produced by cementoblasts that originate from dental follicle cells that are induced by enamel matrix proteins (Slavkin, 1974). Root cementum is primarily responsible for the attachment of periodontal ligament fibres to the enamel surface. In addition, it is also responsible for adjustment of tooth position to continuously changing environment, protection of the dental pulp and repair of enamel defects. Two types of cementum tissue are found in human periodontium, acellular extrinsic fibre cementum (AEFC) and cellular intrinsic fibre cementum (CIFC). They vary in their location, collagen matrix content, structural organization of the matrix, the presence of cells in the matrix, rate of formation, biochemical

composition and degree of mineralization (Lindhe and Karring, 2009; Melcher and Eastoe, 1969; Williams et al., 1992a).

AEFC (primary cementum or acellular cementum) is found on the cervical half to two thirds of the root and approximately 45-60% of the total AEFC completely mineralized. The development of acellular cementum is relative slow. High number of periodontal ligament sharpey's fibre, a type of fibre found in periodontal ligament, insertion into acellular cementum indicates the functional importance in tooth attachment (Fig. 1.1). CIFC (secondary cementum or cellular cementum) is found along the apical half of the root and in the furcation areas. CIFC is also produced in response to resorptive defects and root fractures and is characterized by the presence of intrinsic collagen fibres and cementoblasts trapped in the matrix of cementum (Figs. 1.2 and 1.3). The heterogeneous collagen organization, rapid speed of formation and the presence of the cells in CIFC may be the reason of CIFC being less mineralized compared with AEFC (Lindhe and Karring, 2009; Melcher and Eastoe, 1969; Williams et al., 1992a).

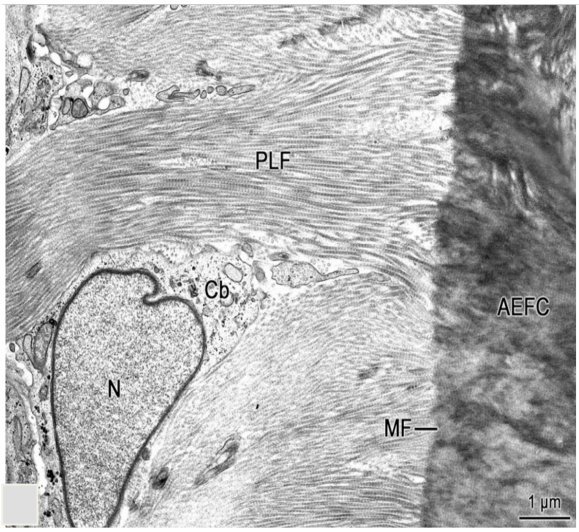


Figure 1.2. Transmission electron micrograph demonstrating the cervical root surface of human tooth. The micrograph exhibits periodontal ligament fibres (PLF) entering the AEFC layer at the mineralization front (MF). Cb, cementoblast; N, nucleus (photo taken from Nanci et al., 2006).

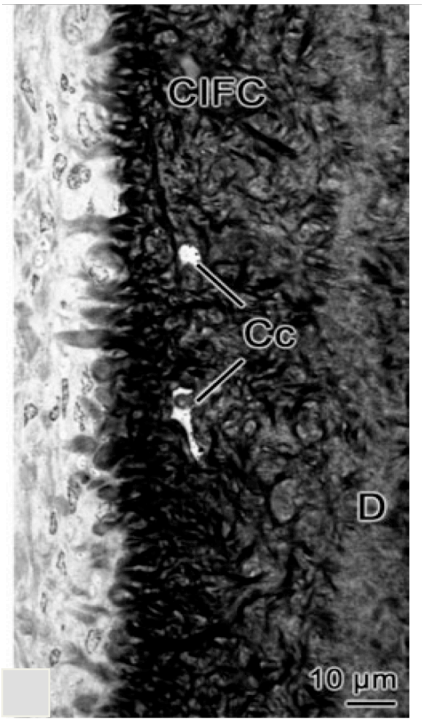


Figure 1.3 Backscattered scanning electron micrographs showing the CIFC in a human premolar. The micrograph demonstrates thick layer of CIFC containing cementocytes trapped in the matrix. Cc, cementocytes; D, dentine (photo taken from Nanci et al., 2006).

Although cementum appears to have different characteristics than bone, such as it does not have blood or lymphatic vessels or nerve innervations or undergo physiological remodeling, the compositions of these tissues are similar. Cementum primarily contains about 50% mineral and 50% organic matrix and predominantly collagen type I constituting 90% of the organic matrix is found in this tissue. Other collagen such as type III, less cross-linked collagen found during development and repair/regeneration, and type XII, a fibril associated collagen that binds to type I and non-collagenous matrix proteins (Lindhe and Karring, 2009; Melcher and Eastoe, 1969; Williams et al., 1992a; Nanci et al., 2006).

1.1.2. Periodontal ligament (PDL)

The PDL is a highly cellular and vascular soft connective tissue of the periodontium interposed between root cementum and the alveolar bone and it anchors the roots of the teeth to the alveolar bone which provides support during mechanical stress or trauma (Hassell et al., 1993; ten Cate, 1994). The PDL also contains sensory nerve innervations which sense various stimuli such as temperature and pressure applied during mastication and pain (Terranova et al., 1990; Lindhe and Karring, 2009).

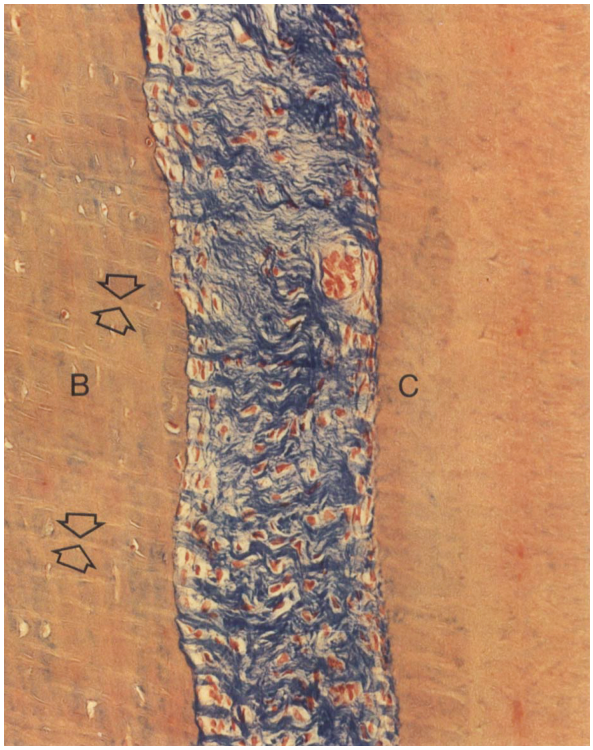


Figure 1.4. PDL interposed between the root cementum (C) and bone (B) showing collagenous fibre network. Note the presence of sharpey's fibres in the bone (arrows). (Photo taken from Beertsen et al., 1997).

Dental follicle cells, derived originally from the inner layer of the ectomesenchyme (Fig 1.4), give rise to the progenitor cells that synthesize cementum, PDL and alveolar bone (Palmer and Lumsden, 1987; Ten Cate et al., 1971; Ten Cate and Mills, 1972; Yoshikawa and Kollar, 1981) (Fig. 1.4). One of the most remarkable features of the PDL is its adaptability to rapidly changing applied force levels that occur during mastication and the capacity to repair and regenerate (Beertsen et al., 1997; Nancy et al., 2006; Lindhe and Karring, 2009). The PDL is also composed of a number of different types of cells and collagenous and non-collagenous extracellular matrix components. The PDL contains fibroblasts, epithelial rest of

malassez, osteoblasts, osteoclasts, monocytes, macrophages, cementoblasts, odontoclasts, neural and endothelial precursors and primitive undifferentiated stem cell-like population. The fibroblasts subpopulations in the PDL are primarily responsible for the synthesis and repair/regeneration of structurally oriented collagen and non-collagenous fibres (Lindhe and Karring, 2009).

These collagen fibres are organized in defined and distinct fibre bundles called principal fibres. The extreme ends of these fibre bundles are embedded into cementum and bone. The embedded portions of the fibres are called sharpey's fibres. Sharpey's fibres in primary acellular cementum are completely mineralized whereas those in bone or cellular cementum are generally only partly mineralized. Other fibre bundles such as gingival ligament fibres, transseptal ligament fibres and elastic fibres are also found in the PDL (Beertsen et al., 1997; Nancy et al., 2006; Lindhe and Karring, 2009).

The epithelial rest of malassez in the PDL are the remnant of Herthwig's epithelial root sheet (HERS). They are found close to the cementum as a cluster of cells which may form epithelium. The function of these cells is not clear but it has been suggested that they may be involved in homeostasis, repair/regeneration of the PDL. Furthermore, primitive stem/precursor cells are important cellular constituent of the PDL, which help rebuilt the PDL and the PDL-associated tissues such cementum, bone, blood vessels and nerves after injury/damage. PDL stem/precursor cells are further discussed in section 1.3.4.

1.1.3. Alveolar bone (AB)

The mammalian skeleton has three distinct origins: the paraxial mesoderm, which gives rise to the axial skeleton; the lateral plate mesoderm, which gives rise to the appendicular skeleton; and the ectoderm (i.e. the neural crest), which gives rise to the facial skeleton, including AB (Chung et al., 2004). Two different mechanisms of bone formation have been reported to occur during morphogenesis (Sommerfeldt and Rubin, 2001).

i) the differentiation of mesenchymal cells directly into osteoblasts, which proceed to form mature bone. This 'intramembranous' bone formation is found during skull development, and also in maxilla (AB of the upper jaw) and mandibular (AB of the lower jaw) morphogenesis;

ii) the differentiation of mesenchymal cells which proceeds via chondrogenic pathway and forms the cartilaginous template of future bones. The hypertrophy of chondrocytes, a process of the enlargement of chondrocytes, of the cartilage is followed by the terminal differentiation of the hypertrophic cells into osteoblasts, ultimately leading to ossification. The whole process is also known as 'endochondral ossification' which primarily occurs during long bone development.

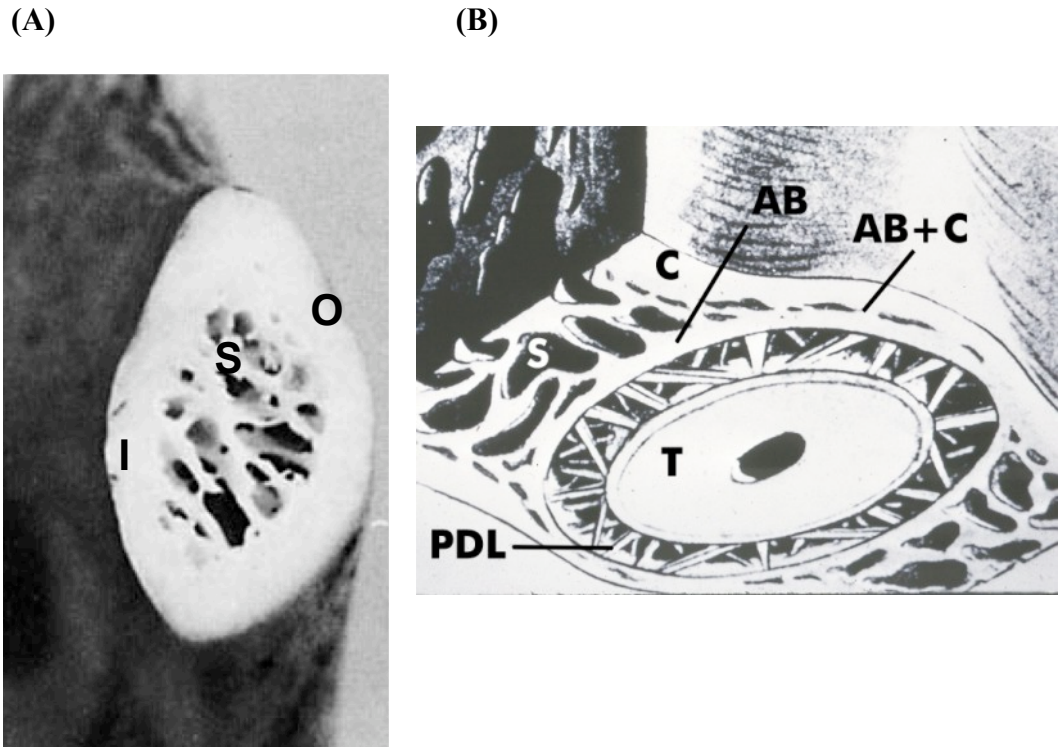


Figure 1.5. Unstained specimen of human mandible showing (A) a cross section and (B) a transverse sections at the middle region of the mandible. The AB houses the roots of erupted tooth (T), PDL while the outer (O) and inner (I) cortical plates of compact bone (C) are found on the right and left sides of the region of the cancellous bone (S), respectively. (Adopted from Hylander, 1975 and http://www.dental.pitt.edu/informatics/periohistology)

A schematic illustration of the structure of AB is shown in Figure 1.5. The maxilla and mandible of adult humans is subdivided into two portions: the AB that houses the roots of erupted teeth and the basal bone that is not involved in housing the roots. The AB consists of the thin part of the bone that forms the alveolar wall of the tooth socket, the inner and outer cortical plates and the cancellous bone. Within the cancellous bone are numerous marrow spaces, with smaller marrow spaces present in the cortical bone (Schroeder, 1986; Sodek and

McKee, 2000). During embryonic development, the intramembranous bone of the maxilla and mandible initially forms from osteoblasts arising from condensing ectomesenchyme in the facial region. As osteogenesis progresses, bone surface become lined by a contiguous layer of osteoblasts that continue to produce bone, which enlarges the dimensions of the trabeculae. Concomitant with the growth of this bone, new mesenchyme condensations arise in neighbouring areas and produce new osteoblasts and new trabeculae, all of which will collectively form the foetal maxilla and mandible (Sodek and McKee, 2000). Although AB has specialized features relating to its functional properties, the cellular compartment and the composition of the extracellular matrix of AB appear to be similar to the other bone tissues, *e.g.*, skull and long bones.

The general functions of AB are to house the roots of teeth and to absorb and distribute ocular pressures generated from tooth contact during mastication. Their most important and unique function is to anchor the roots of teeth to the maxilla and mandible, which form the primary support structure for teeth (Cho and Garant, 2000; Sodek and McKee, 2000). Due to its primary function to house the tooth along with the PDL, bundles of sharpey's fibres penetrate these bone layers and thus, contribute to the characteristic appearance of this bundle bone, although this type of bone exhibiting mineralized sharpey's fibres may not be found at all the sites at the same time because AB undergoes constant homeostasis.

1.1.4. Gingival connective tissue and epithelium

The gingival connective tissue and gingival fibroblasts are considered to originate from mesenchyme (Listgarten, 1972; Luthman et al., 1989). The dentogingival junction, a gingival attachment to the tooth surface and non-keratinized epithelium, is an adaptation of the oral mucosa that comprises epithelial and connective tissue components. The gingival connective tissue is characterized into superficial and deep compartments and epithelium is divided into gingival, sulcular and junctional epithelium. The junctional epithelium plays a pivotal role in sealing-off the periodontal tissues from the oral environment and destruction of junctional epithelial structure leads to periodontal disease.

1.2. Etiology and pathogenesis of periodontitis

Periodontitis is an inflammatory disease of the periodontium that is considered to be caused primarily by a number of different bacterial pathogens and resulting in the progressive destruction of periodontal tissues, possibly by three separate pathways (Nishihara & Koseki, 2004):

i) pathogens directly release proteolytic enzymes that degrade periodontal structures without the intervention of host cells;

ii) pathogens produce products, such as toxins, enzymes and lipopolysaccharides, which may trigger the host cells to produce degenerative enzymes;

iii) pathogens stimulate an immune response resulting in release of a number of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α.

Collagen which is one of the periodontal extracellular matrix (ECM) components appears to be the main target of degradation in periodontitis. Among the host proteases degrading the ECM, matrix metalloproteinases (MMP) and collagenases which seem to be highly associated with tissue destruction and remodeling events in periodontal disease (Nishihara & Koseki, 2004).

The classification of periodontal disease (Armitage et al., 1999) identifies two general clinical manifestations, termed Chronic Periodontitis (CP) and Aggressive Periodontitis (AgP). CP is the most common form of the disease and has been widely investigated (Pihlstrom et al*.,* 2005), with the following characteristics: prevalence mainly in adults; generally slow to moderate rate of destruction of the periodontium; and major role of local factors (e.g. plaque and calculus) as well as smoking and emotional stress (Flemming, 1999). Association with systemic diseases such as diabetes mellitus has also been reported (Pihlstrom et al*.*, 2005). AgP is a severe form of periodontal disease, and affects less than 1% of the population (Loe & Brown, 1991; Saxby, 1987). The characteristics of AgP differ from CP, as follows: high prevalence in young individual; rapid loss of clinical attachment and bone destruction; lack of association with systemic diseases (Tonetti & Mombelli, 1999).

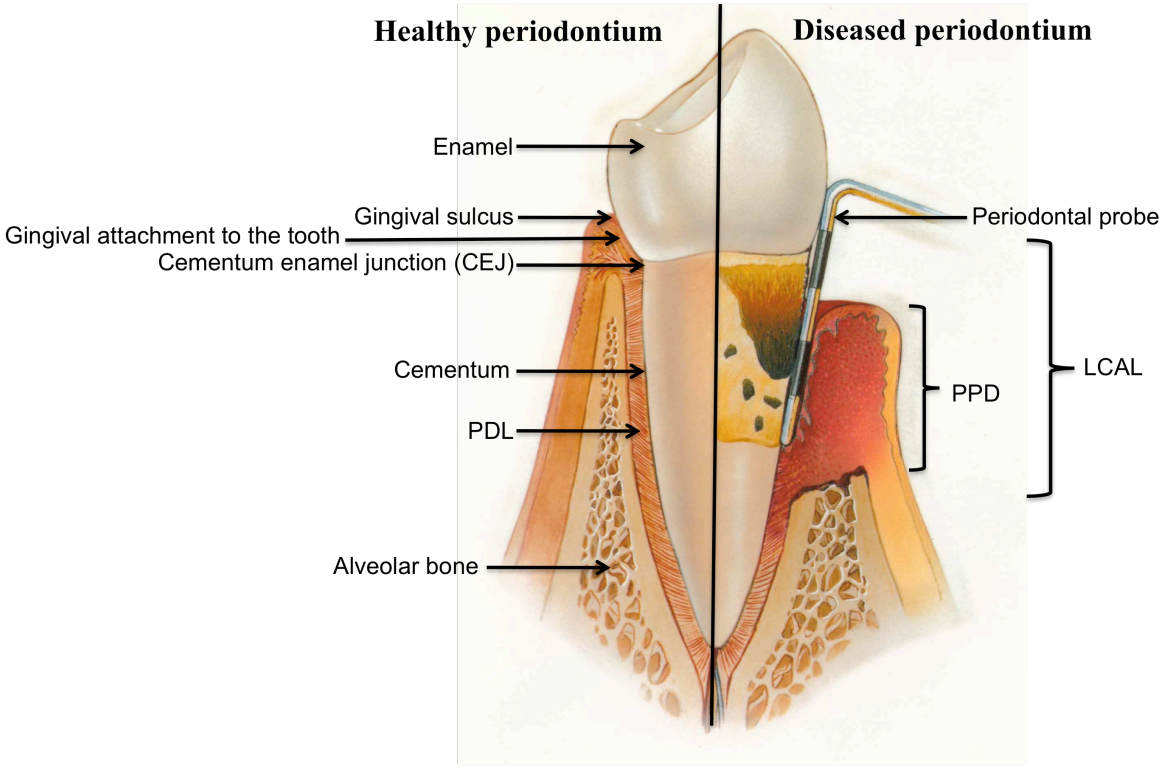


Figure 1.6. The structure of the healthy and diseased periodontium. The healthy periodontium comprises the cementum, PDL, AB and part of the gingivae facing the tooth (creating the space called 'gingival sulcus'). The diseased periodontium is marked by the loss of gingival attachment to the tooth surface, an increase of depth of the gingival sulcus that is known as the periodontal pocket depth (PPD) and destruction of AB. (photo adopted from Oral B laboratory MA, USA)

Chronic gingivitis, characterized by edematous swelling, redness, bleeding of gingival, deepening of gingival sulcus, partial loss of attachment of the junctional epithelium from the dentine and structure change such as enlargement of the free surface area of epithelium leading to an increased exposure to bacterial plaque, can progress into chronic periodontitis. Periodontal lesions can be clinically characterized by apical growth and migration of the junctional epithelium, deepening of periodontal pocket and destruction of the PDL and AB. The degradation of the collagen-rich ECM by the bacterial plaque causes the loss of connective tissue attachment to both AB and root cementum. Furthermore, cementum structure and composition are also altered and superficial accumulation of bacteria and hyper mineralization are also observed (Pihlstrom et al., 2005; Nanci et al., 2006). Since periodontitis causes the destruction of the AB, cementum and the PDL, untreated periodontal lesions may lead to early tooth loss (Pihlstrom et al., 2005; Nanci et al., 2006).

1.3. Periodontal regeneration

1.3.1. Basic principles of tissue regeneration

The main goal of periodontal therapy is to restore healthy and normal periodontal function. The initial phase of periodontal treatment is aimed to eliminate infection and inflammation by removing root surface deposits and to control the bacterial infection by an effective oral hygiene programme (Garrett, 1996). However, if the periodontal defect still persists, surgical procedures are then undertaken to regenerate periodontal tissues.

Tissue regeneration is considered to be dependent on three fundamental elements: i) appropriate signalling molecules; ii) progenitor cells; and iii) sufficient blood supply. Each of these factors is of crucial importance in the healing process and is internally dependent on each other for a successful regeneration of damaged/diseased tissues. Stem/progenitor cells provide the machinery for tissue growth and differentiation. Signalling molecules, such as growth factors and biological mediators, modulate cell proliferation activity and stimulate cell differentiation and the production of ECM. New vascular networks promoted by angiogenic signals provide sufficient blood supply for the new tissue growth (Taba et al., 2005).

1.3.2. Periodontal Access Flap surgery (AF)

AF is a surgical procedure aiming at providing access to the root surface for debridement procedures resulting in reduction of PPD and may attain improved periodontal architecture (Wennstrom et al., 2003). However, the healing which occurs following AF often involves the down-growth of the gingival epithelium (Caton et al., 1980) which attaches to the root surface and often prevents the re-growth of the slower-growing PDL and AB (Ellegaard & Loe, 1971). Due to lack of periodontal connective tissue attachment and insufficient AB at damaged sites the original architecture of the periodontium may not be restored which leads to often an unsuccessful clinical outcome. Nevertheless, AF leads to a significant reduction of PPD (Rosling et al., 1976) and has been widely used as a standard clinical treatment (Cortellini et al., 1998).

1.3.3. Guided Tissue Regeneration (GTR)

GTR is aimed at restoring the original architecture of the periodontal tissue, which is not generally achieved by AF. It involves the placement of an occlusive barrier (resorbable or non-resorbable) into an intrabony defect in such a manner that apical down-growth of the junctional epithelium along the root surface and growth of the gingival connective tissue are prevented (Fig. 1.7) (Karring et al., 1993). At the same time, the occlusive barrier provides adequate space and time for the PDL and AB cells to repopulate the root surface and the bone defect, respectively. GTR was developed for the purpose that the PDL contained progenitor cells required for the regeneration of bone, cementum and PDL and these cells would selectively repopulate the wounded/damaged site that would lead to clinical improvements (Karring et al., 1993).

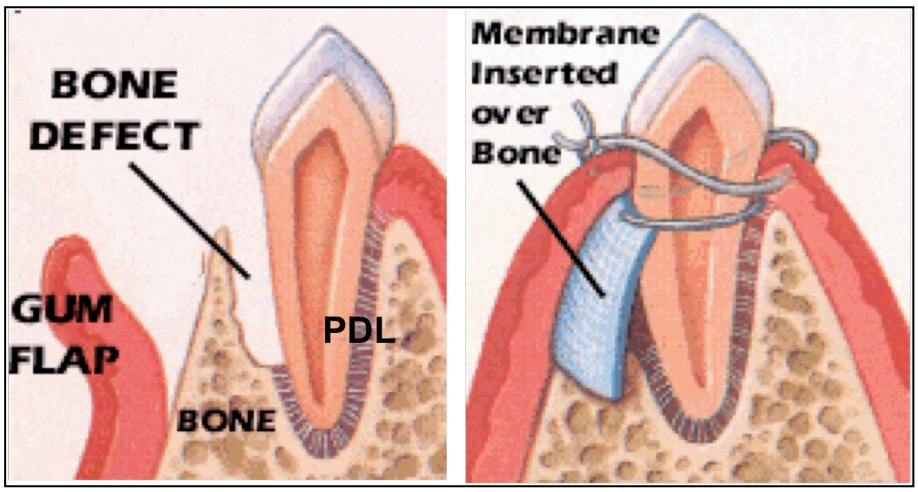


Figure. 1.7. Guided tissue regeneration.

Systematic reviews have suggested that GTR has been more consistently effective than AF at gaining LCAL and reducing PPD in the treatment of periodontal defects of CP patients (Needleman et al., 2005; Murphy & Gunsolley, 2003). However, such trials as well as experimental models have indicated that the extent and predictability of positive clinical outcomes of GTR are limited, and thus there is widespread interest in the development of new therapeutic strategies for promoting more effective periodontal regeneration.

1.3.4. PDL stem/progenitor cells

Stem cells are defined functionally as cells that are able to give rise to at least one differentiated cell type throughout the lifetime of the organism as well as having the unique capacity to self-renew. In contrast to the large majority of the cell population of adult tissues that are committed to a specific function, stem cells are uncommitted and remain as such, until they receive signals from the environment to generate specialized cells (Lemoli et al., 2005). Thus, stem cells are considered to play a key role in tissue homeostasis and the replenishment of cells that have died because of apoptosis, injury and disease (Weissman et al., 2000).

Adult stem cells have been isolated from various types of human and animal tissues including bone marrow (Jiang et al., 2002; Haynesworth et al., 1992), central nervous system (Johe et al., 1996), olfactory epithelium (Barnett et al., 2004), dental pulp (Gronthos et al., 2000), epidermis of the skin (Niemann et al., 2002; Cotsarelis et al., 1990), gastrointestinal track (Potten et al., 1998), blood vessels (Asahara et al., 1997), skeletal muscle (Collins et al., 2005), cornea (Chen et al., 2004), heart (Laugwitz et al., 2005), adipose tissue (Zuk et al., 2001), lung (Wu et al., 2004) and PDL (Singhatanadgit et al., 2009).

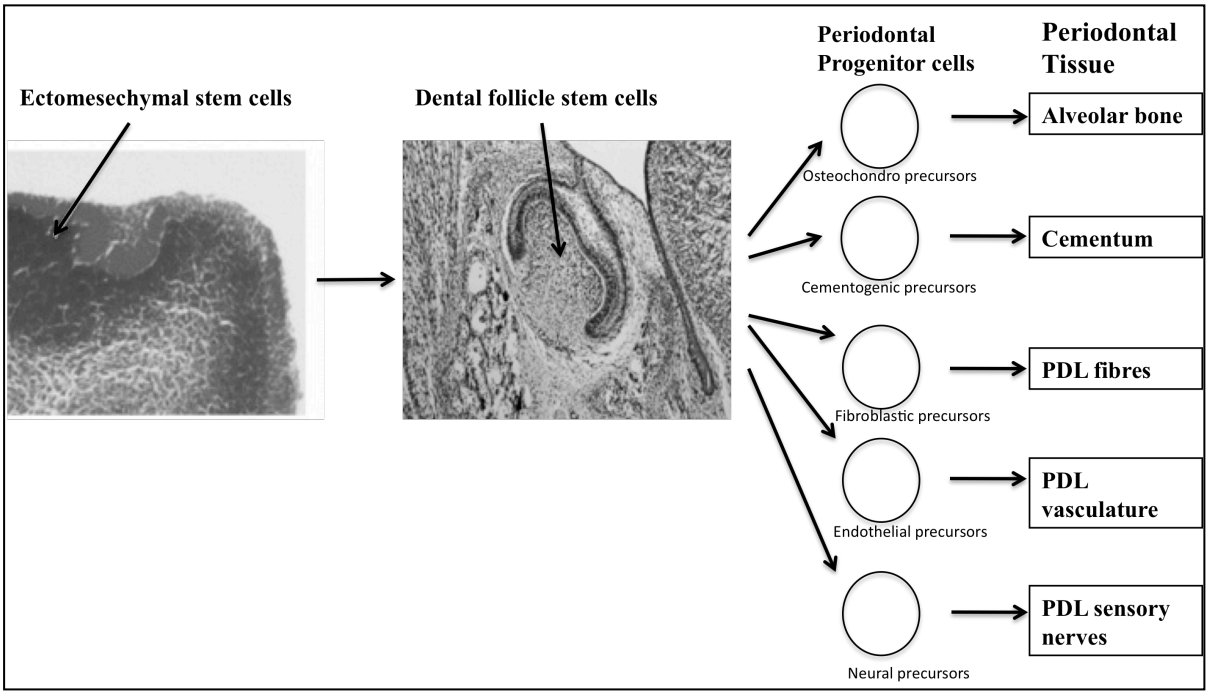


Figure 1.8. Cellular differentiation in the periodontal tissues. (photographs taken from http://www.scielo.br/)

During embryogenesis, the PDL is believed to be formed by cells residing within the dental follicle. These cells are considered to be derived from the ectomesenchyme, as shown in Fig. 1.8. The presence of putative stem cells within the adult PDL was first reported almost 20 years ago (McCulloch et al., 1987), with an increasing number of reports since that time. The most compelling evidence that such cells are present in the PDL was provided by McCulloch (1987), who identified a small population of progenitor cells adjacent to blood vessels within the PDL. These cells demonstrated some typical cytological features of a stem cell-like population, including small size, responsiveness to stimulatory growth factors, slow cell cycle time, higher number of population doublings and colony forming capacity. More recently, several groups have confirmed that the adult PDL contains an adult stem cell-like population that exhibits certain characteristic features of mesenchymal stem cells, and expresses embryonic stem cell-associated antigenic markers such as Oct3/4, nanog and Sox-2 (Huang et al., 2009; Singhatanadgit et al., 2009; Kawanabe et al., 2010). Whether the stem cells isolated from adult human PDL are comparable to bone marrow derived mesenchymal stem cells (BMSC) is still unclear, but it is notable that the putative stem cell marker, STRO-1, used to isolate and purify BMSC, has been shown to be expressed by human PDL cells (Xu et al., 2009; Fujii et al., 2008). In contrast, other reports have suggested that this stem cell-like population residing in adult PDL does not express STRO-1 and may be a more primitive cell population that differs from BMSC (Singhatanadgit et al., 2009; Chen et al., 2006). Nevertheless, adult PDL stem cells share with BMSC the common expression of the perivascular cell marker CD146, alpha-smooth muscle actin and the pericyte-associated antigen 3G5 (Singhatanadgit et al, 2009), suggesting a perivascular origin for these cells as reported previously (McCulloch et al., 1987).

PDL stem cell-like populations have been shown to differentiate into some of the mesoderm-associated lineages (i.e. osteogenic, adipogenic, chondrogenic). Seo et al., 2004 were the first group to isolate PDL ‘stem-like’ cells using ring-cloning technique. These were capable of undergoing osteogenesis and form bone-like mineralized nodules *in vitro* and, moreover, when cultured under adipogenic differentiation conditions, they were also capable of forming lipid-like droplets, a key feature of mature adipocytes *in vitro*. In addition, under chondrogenic differentiation conditions, these cells underwent chondrogenic differentiation, staining positive for proteoglycans (Singhatanadgit et al., 2009, Xu et al., 2009). Thus, while PDL stem cells have been reported to be able to differentiate to various extents into these three mesenchymal lineages, the ‘non-mesenchymal’ vasculogenic, angiogenic, neurogenic

and gliogenic lineage-associated differentiation capability has not been studied previously, despite their physiological, structural and fundamental importance in the PDL. Consequently, although PDL cells have been reported to express a series of embryonic stem cell-associated markers, it is still uncertain whether the PDL contains a primitive pluripotent stem cell-like population.

1.3.5. Mesenchymal lineage differentiation pathways

Osteoblasts, adipocytes, chondrocytes and muscle cells are believed to be derived from multipotent MSC originating in the bone marrow, although alternative pathways have also been reported to be involved in the origin and formation of these different cell types, for example, pericytes, mesenchymal cells adherent to the endothelial layer of vessels, have also been shown to undergo osteogenesis, adipogenesis, chondrogenesis and myogenesis (Schor et al, 1995). PDL stem/progenitor cells have been reported to express several markers associated with BMSC, pericytes and embryonic stem cells, and have been shown to be capable of differentiating along osteogenic, adipogenic and chondrogenic (discussed in 1.3.4). Lineage-specific pathways, as described below.

1.3.5.1. Osteogenic differentiation

Osteogenic differentiation *in vitro* is considered to be the process by which primitive stem cells progress to osteoprogenitor cells and form fully functional osteoblasts. This process involves three stages, with characteristic changes in gene expression during each stage (Stein GS et al., 1996):

(i) proliferation stage: the genes c-fos and c-myc, transcription factors reported to be involved in cell division in response to growth factors/stimuli, are associated with the proliferation stage, while cyclin-B and –E, cell cycle regulation-associated proteins, are up-regulated post-poliferatively (Stein GS et al., 1996);

(ii) ECM production and maturation stage: differential expression of osteoblast-related genes occurs as osteoprogenitor cells differentiate to mature bone cells. This involves the early osteogenesis-associated transcription factor runt-related transcription factor (Runx2) and

ECM enzyme alkaline phosphatase (ALP) genes which are initially up-regulated (although down-regulated later as mineralization progresses), while the secretion of the ECM glycoprotein osteopontin (OP) followed by the expression of other matrix proteins such as bone sialoprotein (BSP) and osteocalcin (OC) (Aubin, 1998b) (BSP is transiently expressed at a very early stage and then increases again in differentiated osteoblasts, while OC is detected mainly during mineralization (Aubin, 1998b)). Thus, the expression of these genes that are closely associated with osteoblast differentiation has been widely used as osteoblast-related markers, for example, Runx2 and OC being early and late markers for osteoblastic differentiation, respectively (Aubin, 2001).

(iii) mineralization stage: the mineralization process involves the secretion of small, round, lipid bilaminar vesicles from osteoblasts at sites of initial mineralization (Manolagas, 2000; Anderson, 2003). The initial crystals of bone mineral are formed within these matrix vesicles, facilitated by the activity of ALP and calcium-binding molecules such as annexin I, which are concentrated at the matrix vesicle membrane. Subsequently, the pre-formed crystals are released through the matrix vesicle membrane and exposed to the extracellular environment containing sufficient calcium and phosphate ions to support continuing crystal growth, with the pre-formed crystals serving as 'seed' nuclei (Anderson, 1989; Anderson, 2003). However, other glycoproteins, such as OC and phosphoproteins, appear to have a regulatory role in preventing excessive mineralization (Anderson, 1989; Ducy et al., 1996). Histochemical staining methods (alizarin red and von Kossa) are widely used to detect the calcium and phosphate ions, respectively, present in the mineralized tissue/cells.

1.3.5.2. Adipogenic differentiation

Several reports have shown that PDL cells are capable of adipogenic differentiation (Huang et al., 2009; Singhatanadgit et al., 2009; Kawanabe et al., 2010; Xu et al., 2009; Fujii et al., 2008), despite the fact that anatomically adipocytes are not detected in PDL tissue. The presence of adipogenic precursors may be due to the presence of a primitive mesenchymal stem cell-like population in the PDL, as discussed in Section 1.3.4. Such cells can be induced to undergo adipogenic differentiation using an 'adipogenic cocktail' containing hormones and growth factors such as insulin, DEX, IBMX, IGF-1, glucocorticoid and c-AMP (Huang et al., 2009; Singhatanadgit et al., 2009; Kawanabe et al., 2010; Xu et al., 2009; Fujii et al., 2008).

The first hallmark of the adipogenesis *in vitro* is a noticeable change from a spindle shape fibroblastic morphology to a spherical shape appearence. This morphological modification is accompanied by marked sequential change in the gene expression categorized into the early, the intermediate and the late phase (Feve et al., 2005; Ntambi et al., 2000). Thus, exposure of confluent PDL stem cells to the adipogenic differentiation cocktail initially induces expression of CCAAT/enhancer binding protein (C/EBP)-β and C/EBPδ, which then activate peroxisome proliferator-activated receptor-γ (PPARγ)-2 and C/EBPα. In addition to C/EBPs and PPARγ2, several other adipogenic transcription factors are also involved, including GATA-binding transcription factors GATA-2, 3, and c-AMP response element binding protein (CREB) which play critical roles in adipogenesis (Feve et al., 2005; Ntambi et al., 2000). During the terminal phase of adipogenic differentiation, activation of the transcription factors leads to increased enzyme activity, for triacylglycerol synthesis and degradation. Expression of lipoprotein lipase (LPL) mRNA has also been shown to be up-regulated by these transcription factors, and is then secreted by mature adipocytes, playing central role in controlling lipid accumulation by hydrolyzing circulating triglycerides (Preiss-Landl et al., 2002).

Terminally differentiated adipocytes also produce low-density lipoprotein (LDL) and high-density lipoprotein (HDL), which can be visualized in a form of lipid-droplets on differentiated adipocytes surface (Preiss-Landl et al., 2002; Murphy et al., 1999). Thus, elevated levels of PPARγ2 and LPL mRNA at early and late stage of adipogenic differentiation, respectively, and production of lipid-like droplets are frequently used as reliable markers of adipogenic-capabilities of precursor cells.

1.3.5.3. Chondrogenic differentiation

Although most of the vertebrate skeleton has been reported to develop from chondrogenic precursors via a process known as ‘endochondral ossification’ as described in 1.1.2, the maxilla and mandibular bone of the skeleton is considered to develop from MSC via a process known as ‘intramembranous ossification’, and therefore chondrogenic precursors are likely to be absent in periodontium including the PDL (Lee et al., 2001). However, it has been reported previously that the PDL contains stem/precursor cell population capable of undergoing chondrogenesis *in vitro* (Huang et al., 2009; Singhatanadgit et al., 2009; Kawanabe et al., 2010; Xu et al., 2009; Fujii et al., 2008).

Much of the current understanding concerning chondrogenic differentiation has been delineated using a micro mass culture system *in vitro*, a three-dimensional, high-density cell culture that is comprised of chondrogenic precursor cells characterized by the potential to differentiate into chondrocyte-like cells (Ahrens et al., 1977). High-seeding density apparently stimulates pre-cartilage cells to mimic the condensation and differentiation events that normally occur during embryonic hyaline cartilage formation *in vivo* (Ahrens et al., 1977). Specifically, precursor cells first undergo condensation, giving rise to aggregates that subsequently differentiate into cartilage nodules (thus simulating the normal progression of *in vivo* chondrogenesis in which mesenchymal condensation precedes observable differentiation (Goldering et al., 2006)). *In vitro* chondrogenesis can be stimulated by addition of growth factors such as TGFβ, IGF-1, EGF and FGF. The sequence of gene expression during chondrogenesis is characterized by the expression of the transcription factor Sox-9, followed by collagen type II and aggrecan, similarly observed *in vivo* and *in vitro* micro mass culture (Goldering et al., 2006; Kulyk et al., 2000). The resulting differentiated chondrocytes secrete proteoglycans (PG) and glycosaminoglycans (GAG) which can be stained with Alcian blue. Thus, the expression of Sox-9, aggrecan, Col2a1 and secretion of PG and GAG by differentiating micro-mass cultures is often accepted as an indication of chondrogenic differentiation *in vitro*.

1.3.6. Non-mesenchymal lineage differentiation pathways

1.3.6.1. Vasculogenic and angiogenic differentiation

Although the PDL contains highly developed vasculature, little is known about neovascularization, a process of new blood vessel formation, by PDL stem cells/precursors *in vivo* or *in vitro*. Neovascularization can be categorized into two general processes: (i) vasculogenesis, the differentiation of angioblast precursors/stem cells into endothelial cells (Flamme et al., 1997); and (ii) angiogenesis, the formation of new capillaries from pre-existing vessels which occurs either by the sprouting of vascular endothelial cells into surrounding tissue or by intussusceptive microvascular growth (D'amore et al., 1987; Flamme et al., 1997).

Although it was previous thought that vasculogenesis occurred exclusively during embryonic development, it is now recognized that the more complex process of neovascularization involves both vasculogenesis and angiogenesis simultaneously in both

adult as well as developing microenvironments (D'amore et al., 1987; Flamme et al., 1997). In general, although the molecular regulation of vasculogenesis and angiogenesis differ, various vascular endothelial growth factors (VEGF-A, B, C and D) play pivotal roles in both of these processes (Larrivee et al., 2000; D'amore et al., 1987; Flamme et al., 1997; Ambrosi et al., 2005). It has been shown that VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2) knockout mice die within 8.5-9.5 days of embryonic development suggesting, VEGF receptors play pivotal role in vasculogenesis during embryonic development. In vasculogenesis, initially VEGF-A is responsible for VEGFR-2 up-regulation followed by VEGFR-1 stimulation. Similar to its role in vasculogenesis, VEGF-A and C are also responsible for the regulation of angiogenesis. However, while VEGFR-2 receptor is dominant during early vasculogenesis, VEGFR-1 receptor is prominent during remodeling of the primary vasculature and subsequent angiogenesis (Hanahan et al., 1997). Numerous studies have shown *in vitro* that these VEGFs promote migration, proliferation and tube formation by endothelial cells (Larrivee et al., 2000; D'amore et al., 1987; Flamme et al., 1997).

Unlike VEGF receptors, angiopoietin (Ang) receptors tie-1 and 2 have been shown to be expressed by endothelial cells only. Tie-1 and tie-2 knockout mice undergo severe vascular malformations and die during embryonic development (Larrivee et al., 2000; D'amore et al., 1987; Flamme et al., 1997; Hanahan et al., 1997). Differentiated endothelial cells express von willebrand factor, a blood glycoprotein produced in specialized endothelial cell vesicle weibel-palade bodies, VE-cadherin and PE-CAM (CD31), endothelial cell junction/adhesion proteins (Cockerill et al., 1995; D'amore et al., 1987; Flamme et al., 1997). Expression of these markers along with morphological evidence of tube-like structures *in vitro* and *in vivo* is often used as a good indication for vasculogenesis and angiogenesis.

1.3.6.2. Neurogenic differentiation

The PDL receives dense sensory nerve innervations from both the trigeminal ganglion and mesencephalic trigeminal nucleus that are responsible for touch, pressure and pain sensations. They are regarded as an important component of the sensory system for controlling the jaw movement. At least two types of sensory receptors have been recognized in the PDL; (i) nociceptive free nerve endings; and (ii) specialized mechanoreceptive endings, also known as 'ruffini endings'. Moreover, previous studies have suggested that mature periodontal ruffini

endings regenerate rapidly following trauma or injury (Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997), indicating that they have a high potential for neuroplasticity and possibly contain neural stem/precursor cells with the capacity to regenerate new nerves.

The PDL is also known to contain myelinating and non-myelinating glial-associated Schwann cells known as lamellar or terminal Schwann cells, which play a crucial role during neural development and regeneration by guiding developing/regenerating nerve axons (Bhatheja et al., 2006). Schwann cells stain positive for glial markers such as S-100β and GFAP. Protein gene product 9.5 (PGP9.5), also known as ubiquitin carboxy-terminal hydrolase L1 (uclh1), abundantly found in nerve cells and is involved in protein turnover, in nerve cells, and used as a marker for the identification of periodontal ruffini endings in PDL tissue (Nandasena et al., 2007).

It has previously been suggested that neural precursor may be present in PDL tissue and may be the cellular source of periodontal nerves and glial cells observed *in vivo* although PDL cells have hitherto not been reported to undergo neurogenesis or gliogenesis *in vitro* or *in vivo.* Well-developed differentiation systems are now available to investigate the pathways of neurogenesis and gliogenesis *in vitro*, mainly using laminin, a basal membrane protein which has been found in almost all the cells and tissue and involved in cell adhesion, proliferation and differentiation. Two major culture systems used for neurogenesis and gliogenesis *in vitro* are:

(i) a free floating neurosphere serum-free culture in which putative neural progenitors are placed into non-adherent plastic culture dishes and allowed to form aggregates in the presence of growth factors such as bFGF and EGF. After several disaggregation and transfers of the cell spheres, they are dissociated and seeded on laminin-coated plates under neurogenic or gliogenic inductive conditions to differentiate the cells into mature nerves and/or glial cells (Hermann et al., 2004).

(ii) confluent monolayer cultures of cells seeded onto laminin-coated culture dishes and the monolayer of adherent cells incubated until 80-90% confluency, at which neurogenic or gliogenic growth factors are added to differentiate the cells into mature nerve or glial cells (Donato et al., 2007).

Neuronal differentiation *in vitro* is stimulated by the addition of β-mercaptoethanol and forskolin (which raises cAMP levels), all-trans-retinoic acid, brain-derived growth factor

(BDNF), neurotrophine-3 (NT-3), bFGF and EGF whereas, the glial differentiation pathway is induced by foskolin and a growth factor hereguline-β2. Morphologically neurons exhibit a cell with long 'axonal' projection and several dendritic projections with a small neuronal body known as a 'soma', whereas glial cells can be visualized as a star-shaped cell with 3-10 dendritic projections known as 'astrocyte', with 20-30 dendritic projections known as 'oligodendrocytes' and with 2 dendritic projections known as 'Schwann cells'. Immunostaining using neuron-specific-βIII tubulin and microtubule-associated protein-2 (MAP-2) antigens as early neural cell filament markers, and PGP9.5, neurofilament M/H/L and enolase as late neuronal antigens have been widely used. GFAP, oligodendrocytic-specific O4 and S-100 markers are used for the immunodetection of glia-associated cells such as astrocytes, oligodendrocytes and Schwann cells, respectively (Hermann et al., 2004; Donato et al., 2007; Cora et al., 2009). Expression of these markers together with morphological evidence of neural and glial-like cells *in vitro* and *in vivo* often used as the evidence of neurogenic and gliogenic differentiation.

1.3.7. Growth factors/biological mediators for periodontal regeneration

A number of growth factors and biological mediators have been used as an alternative to the surgical treatment approach for periodontal regeneration (Lee et al., 2009; Shirakata et al., 2010; Giannobile et al., 1996; Cornelini et al., 2003; Camelo et al., 2003; Jones et al., 2006; Hammarstrom, 1997; Kao et al., 2009; Brett et al., 2004; Zhao et al., 2002; Strayhorn et al., 1999; Chien et al., 1999; Takayama et al., 1997). These include platelet-derived growth factors (PDGF), IGF-1, bFGF, TGF-β, bone morphogenetic proteins (BMPs), and EMP, which have been shown to have stimulatory effects to some extent on periodontal regeneration *in vivo* (Lee et al., 2009; Shirakata et al., 2010; Giannobile et al., 1996; Cornelini et al., 2003; Camelo et al., 2003; Jones et al., 2006; Hammarstrom, 1997) and variable effects on periodontal cell proliferation and differentiation activity *in vitro* (Kao et al., 2009; Brett et al., 2004; Zhao et al., 2002; Strayhorn et al., 1999; Chien et al., 1999; Takayama et al., 1997). Table 1 presents a list of growth factors and their effects on PDL cells, cementoblasts and osteoblasts *in vitro*. Table 1 with EMP having greater overall stimulatory effect on periodontal cells *in vitro* compared with the effects of other growth factors on periodontal cells. Moreover, EMP has been shown to stimulate PDL, cementum and AB regeneration *in vivo,* in animals and in humans, as described in 1.4.3.

Table 1. Effects of growth factors and EMP on PDL cells (PDL), cementoblasts and osteoblasts (OB) *in vitro* (Kao et al., 2009; Brett et al., 2004; Zhao et al., 2002; Strayhorn et al., 1999; Chien et al., 1999; Takayama et al., 1997)

Growth factors	Cell migration			Cell proliferation			Cell differentiation			Matrix gene expression		
Cell types	**PDL**	**CM**	**OB**	**PDL**	**CM**	**OB**	**PDL**	**CM**	**OB**	**PDL**	**CM**	**OB**
PDGF	++	**?**	++	+++	+++	+++	**-**	**-**	**-**	++	**0**	+
IGF-1	++	**?**	++	+	++	+	**-**	**?**	+	+	+/-	++
FGF-2	+++	**?**	**0**	+++	**?**	+++	**-**	**?**	**-**	+/-	**?**	+/-
TGF-β	+	**?**	++	++	++	++	+	**-**	+/-	++	+/-	++
BMPs	**?**	**?**	++	**0**	**-**	+	**?**	+	+++	**?**	++	++
EMP	+++	**?**	++	+++	+++	+++	+	+	+	+	+++	+++

(-) Inhibitory effect, (0) no effect, (+) mild stimulatory effect, (++) modest stimulatory effect, (+++) strong stimulatory effect, (?) unknown effect

1.4. Enamel Matrix Proteins (EMP)

1.4.1. Secretion of EMP

Enamel, a unique and highly mineralized ectodermal tissue covering vertebrate teeth, is synthesized and secreted by specialized cells called ameloblasts. During the process of enamel development ameloblasts secrete EMP that binds to hydroxyapatite to structure the enamel and to modulate crystal growth (Deutsch et al., 1995; ten Cate et al., 1996; Heritier et al., 1982; ten Cate et al., 1996). The secretion of EMP can be observed during three distinct stages of enamel formation: (i) the pre-secretory stage (ten Cate et al., 1996); (ii) the secretory stage (also called forming stage) (Heritier et al., 1982); and (iii) the maturing stage (also called the secondary mineralization stage). Although the major biosynthesis and secretion of EMP has been reported to take place in the secretory stage, the full-length amelogenin and its splicing forms (e.g., LRAP), major protein components of EMP, are also secreted in the early pre-secretory and late maturation stages (Deutsch et al., 1995). In addition to EMP, growth factors such as BMP and TGFβ have also been shown to be secreted during enamel development (Deutsch et al., 1995; Ten cate et al., 1996; Heritier et al., 1982; ten Cate et al., 1996). Within hours after secretion, progressive proteolytic clipping of amelogenin peptides has been shown to give rise to breakdown products of amelogenin (including TRAP), and secreted EMP components have also been shown to be gradually degraded by enzymatic digestion (Deutsch et al., 1995; Deutsch, 1989), suggesting that the secretion of EMP components varies between different stages of enamel development.

EMP secreted by ameloblasts has been shown to play a crucial role in enamel formation and biomineralization (Deutsch et al., 1995). Other findings indicate that EMP also has a function other than enamel development, such as in dentine root formation (Hammarstrom et al., 1997a; Heijil et al., 1996). The involvement of EMP in root cementum formation was first proposed by Slavkin (1974), who suggested that Hertwig epithelial root sheath (HERS) cells produce a basement membrane containing EMP that directs the induction of cementoblast differentiation from dental follicle cells (Slavkin, 1974). Several hypotheses explaining the role of EMP in root cementum formation have been proposed: (i) it is involved in the attachment of root dentine; (ii) it initiates cementogenesis; (iii) it serves as an inducer of dental follicle cell differentiation into cementoblasts (Slavkin, 1974). EMP may also have a significant role in periodontal development and regeneration, for example, Hammarstrom (1997a) demonstrated that porcine EMP placed in experimental cavities created in monkeys by extracting incisors induced the formation of a tissue identical to acellular, extrinsic fibre

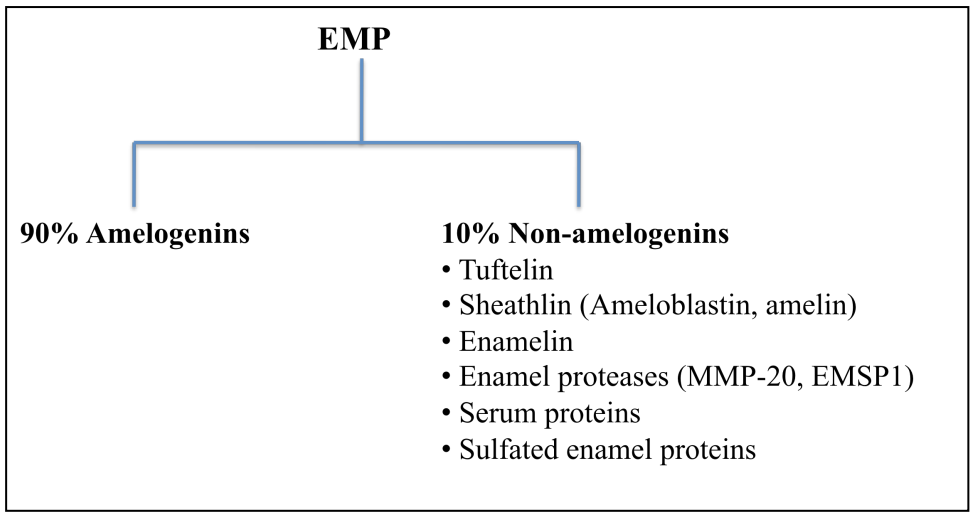
cementum (Hammarstrom et al, 1997a). Thus, treatment with extracted crude EMP was found to result in 60-80% formation of new cementum and bone in such surgically produced periodontal defects (Hammarstrom et al., 1997a).

The major EMP components secreted by ameloblasts, have been partly identified and characterized, and comprise the hydrophobic amelogenins, sheathlins, the acidic tuftelin, a high molecular weight enamelin and various isoforms of these proteins. However, as described above, the content of EMP varies between different stages of enamel development and it is therefore likely that there are also variations between different EMP preparations. A commercial product derived from EMP, called Enamel Matrix Derivatives (EMD; Emdogain®) and designated as FDA approved 'material' for periodontal regeneration since 1997, has been obtained via a rigidly controlled industrial process of purification and heat-treatment of an acidic extract of developing enamel from six-month-old piglets. In this thesis, EMP designation refers to the relatively crude and non-heat-treated mixture of proteins that are obtained from developing porcine enamel, whereas EMD designation refers to the commercially prepared heat-treated lyophilized proteins isolated from porcine enamel during a specific stage of development.

1.4.2. Composition of EMP

The major components of EMP are **amelogenins**, a family of hydrophobic proteins that accounts for more than 90% of the organic matter of the enamel matrix (Brookes et al., 1995). The amelogenins have been remarkably conserved through evolution, suggesting that they may be of critical biological importance (Brookes et al., 1995). Other abundant 'non-amelogenin' components in EMP include **enamelins** (Brookes et al., 1995), **tuftelin** (Deutsch et al., 1991) and **sheathlin** proteins (Hu et al., 1997). As shown in Fig. 1.9, these four matrix proteins, amelogenins (Hu et al., 1996), enamelins (Hu et al., 1997b), tuftelin (Deutsch et al., 1991) and sheathlins (also called ameloblastin or amelin) (Hu et al., 1997a), and also 2 enzymes matrix metalloproteinase (MMP)-20 and enamel matrix serine proteinase (EMSP)-1 (Fukae et al., 1987), are present in EMP.

Figure 1.9.



Composition of EMP

Investigation of possible growth factors in EMP concluded that they were not present in such preparations (Gestrelius et al, 1997b), but other studies indicated the presence of low levels of TGF-β1 and BMP-2 (Kawase et al., 2001; Iwata et al., 2002). These growth factors have been shown to be secreted along with EMP during enamel developmental process (Deutsch et al., 1995; ten Cate et al., 1996; Heritier et al., 1982; ten Cate et al., 1996) and, as noted above, their can also vary between different stages of enamel development and thus differ between preparations. However, because of this, since 2005 the commercially available preparation EMD, which is heat-treated and unlikely to have any enzyme or growth factor activity, has been widely used.

1.4.2.1. Amelogenins

Amelogenin is a major protein component of EMD, accounting for approximately 90% of the protein secreted by ameloblasts (Gestrelius et al., 2000). It is a hydrophobic protein, rich in proline, glutamine, leucine and histidine and exhibiting a very high degree of sequence homology (>80%) among the higher vertebrates examined (Fincham et al., 2000). The multiple amelogenin peptides present in EMD are the products of alternative splicing of the amelogenin gene and also of proteolytic processing of the parent proteins, as shown in Fig. 1.10. Although the primary transcript of the amelogenin gene is highly conserved between species, the alternative splicing forms vary in number and structure between species. For example, 5 different splicing variants of amelogenin have been identified in human, whereas in the mouse there are at least 14 different splicing isoforms. In pig, 4 different splicing forms

of amelogenins have been identified, each translated into the 4 corresponding amelogenin peptide isoforms (27, 25, 18 and 6.5 kDa), as shown in Fig. 1.10. The smallest splicing variant is a 6.5 kDa (56-amino acid) leucine-rich amelogenin peptide (LRAP) containing the N-terminal (33 amino acids) and C-terminal (23 amino acids) sequences of the full-length amelogenin (tan Cate et al. 1996; Fincham et al., 1994).

The 25 kDa splicing variant of amelogenin is the main source of the majority of the proteolytic products of amelogenin. It is converted either to the 20 kDa amelogenin, which is the most abundant amelogenin in secretory porcine enamel and in EMD, and to the 23 kDa amelogenin. It has been shown that the 20 kDa amelogenin is further processed proteolytically to 5.3 and 5.1 kDa tyrosine rich amelogenin peptides (TRAP) (Fincham et al., 1994).

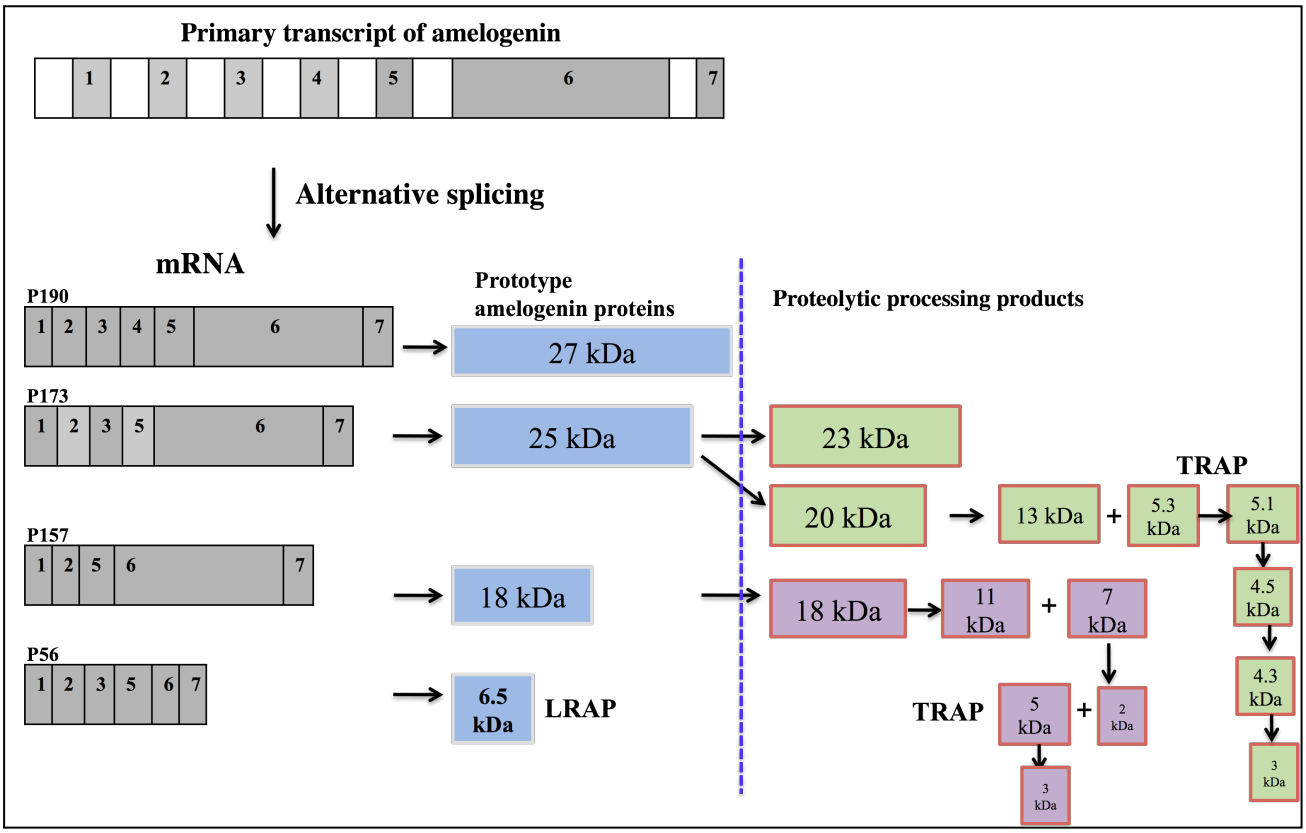


Figure 1.10. A schematic diagram of extracellular porcine amelogenin mRNA splicing and proteolytic processing. Numbers in gray boxes represent exons.

The N-terminal of TRAP has been shown to be identical to the N-terminal of the 20 kDa amelogenin. Other pathways of proteolytic degradation of the 18 kDa splicing variant also produce a 5 kDa TRAP as well as additional uncharacterized proteins. All these different forms of amelogenins shown in Fig. 1.10 have been reported to be present in EMD (Fincham et al., 1994), although their precise functions are not yet known.

1.4.2.2. Non-amelogenins

Enamelin is the largest enamel protein and is concentrated along the secretory face of the ameloblasts. Following secretion by ameloblasts, it is processed to other low-molecular weight proteins associated with progressive enamel mineralization, and is also believed to have a role in enamel biomineralization (Brookes et al., 1995).

Tuftelin is an anionic non-amelogenin enamel protein first fully characterized by Deutsch et al., 1991. It is expressed as early as the bud stage of tooth development and may be involved in crystal formation of the tooth and also in ameloblast differentiation and/or extracellular matrix secretion (Paine et al., 2000).

Sheathlin (also known as *ameloblastin* and *amelin*) represents 5% of the non-amelogenin mRNA and has a domain homologous with the α2β1 integrin recognition site present in collagen type I (Cerny et al., 1996). It is localized in ameloblasts and in the sheath space in enamel, and may have a role in enamel biomineralization. The sheathlin gene is localized in the region where the autosomal dominant Amelogenesis Imperfecta gene has been identified (Forsman et al., 1994), again suggesting that this protein is important for enamel formation.

1.4.3. Role of EMD in periodontal regeneration

1.4.3.1. *In vivo* studies

The regeneration of acellular cementum was first demonstrated in monkeys, in which attachment to dentin was observed after 8 weeks of treatment with crude porcine EMP (Hammarstrom et al, 1997a), whereas healing of the control sites that were not treated with EMP were reported to be uneven with a thick layer of a cellular, hard tissue that was poorly attached to the denuded dentin (Hammarstrom et al., 1997a). Another study of buccal dehiscence defects in monkeys reported 60-80% regeneration of the cementum after application of whole EMP or an acid extract of EMP (both crude non-commercial preparations) to the denuded root surface (Hammarstrom et al., 1997b). However, compared with cementum regeneration, the extent of new bone formation in this study was much less (Hammarstrom et al., 1997b). These studies thus suggested that although crude EMP could be a useful mediator for cementum regeneration, the bone regenerative activity of this material was uncertain.

Several animal studies have been performed to compare histological outcomes following flap surgery with and without EMD treatment. 'Critical-size fenestration', 'infrabony' and 'furcation recession' were produced surgically in the AB of monkeys, and then treated for 5 months with flap + EMD or a coronally repositioned flap as a control (Donos et al., 2003; Sculean et al., 2000a; Sculean et al., 2000b; Araujo et al., 1996; Gkranias et al., 2010). The results demonstrated that one out of three EMD-treated defects exhibited complete closure with regenerated bone while the defects in the other two animals showed only partial closure, but all three animals treated with EMD exhibited fully regenerated PDL and cementum with inserting PDL fibres. In the control specimens in which the defect was not treated with EMD, the defect was found to be remained open with very little if any PDL, cementum and bone regeneration, indicating that EMD may have had stimulatory effects on PDL and cementum regeneration, but again bone regeneration in the presence of EMD was inconsistent in this animal model.

The osteoinductive activity of EMD has been examined using an immune-deficient (nude) mice in a heterotopic (outside the skeletal tissue) bone formation model (Boyan et al., 2000; Kawana et al., 2001). Although no bone formation was observed when EMD alone (used at a significantly higher concentration (4 mg) than the recommended concentration for clinical use (300 μg)) was implanted into muscle, when the same amount of EMD was

implanted together with osteoinductive demineralised freeze-dried bone allograft (DFDBA), enhanced bone stimulation was observed compared with the osteoinductive DFDBA alone. These results thus indicated that high concentrations of EMD might increase the osteoinductive properties of the bone graft material. In contrast, a study evaluating the osteoinductive properties of EMD and deproteinized bovine bone mineral (DBBM) in the muscle of rats reported that EMD alone, DBBM alone or both combined did not stimulate bone (Donos et al., 2006). The lack of osteogenic potential of EMD has also been demonstrated in two established models of bone regeneration; the 'dome' model in the mandibular ramus of rat and the critical-size calvarial defect (Donos et al., 2004; Donos et al., 2005), again indicating the inconsistent effects of EMD on bone regeneration, possibly due to varying experimental conditions including different animal models/defects, varying age and healing conditions.

In humans, using a mandibular incisor extracted for orthodontic reasons, a buccal dehiscence defect was created and treated with EMD (Heijl et al., 1997). After 4 months of healing, the results showed formation of new acellular cementum, new PDL with inserting and functionally oriented collagen fibres, and associated AB covering the area of the defect (Heijl et al., 1997). In contrast, another study observed that only 2 out of 7 intrabony defects treated with EMD developed fully regenerated PDL, cementum and AB, whereas the other 5 defects were characterized by insufficient bone regeneration (Sculean et al., 1999). Similarly, another clinical study observed that only 3 out of 10 intrabony defects exhibited full periodontal regeneration (PDL, cementum and AB) (Yukna and Mellonig, 2000). A number of clinical studies carried out to evaluate the effects of EMD on periodontal soft tissue wound healing also showed discrepancies Wennstrom et al., 2002; Hagennars et al., 2004). For example, periodontal wounds treated topically with EMD exhibited rapid and complete healing and PDL regeneration, compared with the control sites that were not treated with EMD which exhibited only partial healing and PDL regeneration (Wennstrom et al., 2002). In contrast, it was shown that a test patient group treated with EMD exhibited only partial PDL regeneration, similar to the control group that did not receive EMD (Hagennars et al., 2004). The reason for these discrepancies is not yet clear, but it may partly be due to varying experimental conditions, including age of patients, severity of periodontal and other systemic diseases (e.g., diabetes), life style (i.e., eating habits, smoking) and oral hygiene/removal of bacterial plaque prior to regenerative procedures.

In summary, in the presence of EMD 'true' periodontal regeneration, which includes PDL, attachment of acellular cementum and AB regeneration, is not yet predictable and reproducible. In addition, the establishment of new BV and the formation of proprioceptive sensory nerve innervations are also key features of 'true' periodontal healing/regeneration (Imai et al., 2003), but the effects of EMD on regeneration of BV and nerves during periodontal regeneration have not hitherto been investigated *in vivo* or *in vitro*.

1.4.3.2. *In vitro* studies

A number of investigations have been carried out to clarify the effects of EMD on periodontal regeneration (Schwartz et al., 2000; Tokiyasu et al., 2000; Jiang et al., 2001; Ohyama et al., 2002; Yoneda et al., 2003), using several different types of cell *in vitro*. For example, Schwartz (2000) showed that EMD induced proliferation and stimulated the osteogenic markers OC and ALP in a normal human osteoblast cell line although in the same study it was also observed that EMD induced proliferation but suppressed OC and ALP expression of a pre-osteoblast cell line 2T9 (Schwartz et al., 2000). Moreover, it was also shown that EMD suppressed proliferation but induced OC and ALP expression by the osteosarcoma cell line MG63 (Schwartz et al., 2000), whereas EMD suppressed OC gene expression and mineralization by the osteoblast and cementoblast cell lines OCT-1 and MC3T3 Tokiyasu (2000). In addition, EMD enhanced the proliferation of primary osteoblasts but stimulated the bone resorption markers IL-6 and prostaglandin G/H synthase 2, while failing to stimulate OC gene expression (Jiang et al., 2001). The studies noted above clearly indicate discrepancies in the effects of EMD on osteogenic differentiation *in vitro*, possibly because of the use of different cell types, varying culture conditions and varying length of incubation time.

Similarly, in studies of other lineages, Ohyama (2002) showed that the osteogenic and chondrogenic markers (ALP, OC, Col X) of a pluripotent mesenchymal cell line C2C12 were stimulated by EMD, whereas EMD suppressed the myogenic markers (myoD1 and desmin), indicating that at least some components in EMD were capable of differentially regulating these pathways in C2C12 cells. In contrast, Dean (2002) showed that although EMD increased proliferation of pre-mature chondrocytes, it inhibited chondrogenic differentiation as measured by ALP activity and had no measurable effects of collagen synthesis, proteoglycan secretion and TGF-β production. Another study using pre-chondrocytes also

concluded that EMD up-regulated proliferation but failed to increase ALP activity and PGE_2 production (Yoneda et al., 2003), again indicating discrepancies in the effects of EMD on chondrogenesis.

Several studies using PDL cells to investigate the effects of EMD on osteogenesis found that it stimulated ALP activity and bone-like nodule formation under growth as well as differentiation conditions (Gestrelius et al., 1997; Van der Pauw et al. 2000). Similarly, in a study by Nagano (2004), expression of ALP mRNA and ALP activity were dose-dependently increased when PDL cells were treated with EMD under growth conditions. In contrast, Cattaneo (2003) observed the lack of ALP activity stimulation in human PDL cells cultured on EMD-coated tissue culture dishes. Hakki et al., (2001) showed that EMD suppressed OC gene expression and blocked mineralization of murine dental follicle cells under differentiation conditions *in vitro*. Thus, in some cases EMD appeared to stimulate osteogenic marker genes and terminal osteogenic differentiation, indicating the possible presence of osteoinductive component(s) in EMD. However, other contradictory results have demonstrated the lack of consistency between studies of the effects of EMD on osteogenic differentiation, possibly due to differences in cell type and varying experimental conditions (eg, treatment with EMD on cell monolayers, culture of cells on EMD-coated tissue culture dishes and differences in culture media).

Because of the discrepancies observed on the responses of PDL and other bone-forming cells *in vitro,* several attempts have been made to fractionate freshly isolated porcine EMP in order to evaluate the specific component which could have osteoinductive activity. Iwata (2002) fractionated EMP from developing porcine teeth and reported the osteoinductive fraction contained mainly 20 kDa proteins. This fraction enhanced ALP activity and mineralized nodule formation and up-regulated OC, BSP and ALP gene expression in the mouse bone marrow stromal cell line ST2. However, the methodology used in this study could not exclude the possibility that the osteoinductive fraction might contain additional low-molecular weight amelogenins and other proteins. In addition, crude extracts of EMP derived from developing pigs are known to contain growth factors such as BMPs and TGF-β, which may have also contributed to the osteoinductive effects observed in this report (Iwata et al., 2002). Nagano (2006) used crude EMP fractions to look at osteogenic differentiation by ST2 cells and concluded that the crude EMP contained TGF-β1, again demonstrating the importance of using more purified EMP components to examine biological activity. There is therefore a need for less heterogeneous and more consistent preparations of EMP, such as

EMD, in order to understand the mechanism(s) by which specific components influence PDL cell differentiation in vitro and may lead to successful regeneration of damaged periodontal soft and hard tissue, BV and nerves *in vivo*.

1.5. Interaction between EMD and target PDL cells

Endocytosis is the main mechanism whereby macromolecules are internalized into mammalian cells and targeted to specific intracellular vesicles, ultimately to lysosomes where they are usually degraded (Mukherjee et al., 1997). The process is generally initiated by the binding of an exogenous ligand, which can include morphogens, growth factors, cytokines, lipids, some pathogenic bacteria and certain viruses, to transmembrane receptors (Mukherjee et al., 1997). The ligand-receptor complex is then internalized via one of three distinct pathways, involving: (i) clatherin-coated vesicles; (ii) caveolae-coated vesicles; (iii) clatherin- and caveolae-independent vesicles. Clatherin-mediated endocytosis occurs via clatherin-coated pits, 150 nm invaginated structures on the plasma membrane in which the transmembrane receptors are concentrated and which can occupy as much as 2-4% of the surface of cells such as fibroblasts (Mukherjee et al., 1997; Conner and Schmid, 2003). Ligand-receptor complexes become located within the clatherin-coated pits which then pinch-off to form clatherin-coated vesicles, subsequently transporting the complex inside the cell to specific intracellular compartments (Fig. 1.11) (Mukherjee et al., 1997; Conner and Schmid, 2003).

In certain types of cell including endothelial cells, adipocytes, smooth muscle cells and fibroblasts, macromolecule internalization can also occur via caveolae-mediated endocytosis. This involves the invagination of caveoli protein-rich plasma membrane vesicles 50-100 nm in diameter, in which ligand-receptor complexes are also concentrated (Anderson, 1998). Macromolecules such as albumin, certain signalling molecules (e.g. the glycophosphatidylinositol (GPI)-linked protein CD59) and simian virus-40 are known to be internalized via this caveolae-dependent endocytic pathway (Anderson, 1998). Although it has been suggested that macromolecules internalized via caveolae-mediated endocytosis may have a different intracellular fate compared with clatherin-mediated endocytosis, the exact intracellular trafficking events and their physiological relevance are not yet clear. Another mechanism of internalization, involving neither clatherin nor caveolae endocytosis, is also not well characterized (Conner and Schmid, 2003), but is considered to be involved in uptake of some glycosphingolipids and bacterial toxins such as cholera and shiga (Conner and Schmid, 2003; Anderson, 1998).

Endocytosis via clatherin-coated vesicles has been the most widely studied process of ligand internalization. Soon after the clatherin-coated vesicles pinch-off from the plasma membrane, the clatherin coats begin to disassemble and the vesicles fuse with ‘early

endosomes', a membrane-bound intracellular compartment identified by the early endosome antigen-1 (EEA-1) and Rab5 protein (Mukherjee et al., 1997; Conner and Schmid, 2003) (Fig. 1.7). These early endosomes may be transported back directly to the plasma membrane (direct recycling) or transported via 'recycling endosomes' to the perinuclear trans-Golgi network (TGN), identified by the antigen markers TGN38 and 58K (Mukherjee et al., 1997; Conner and Schmid, 2003).

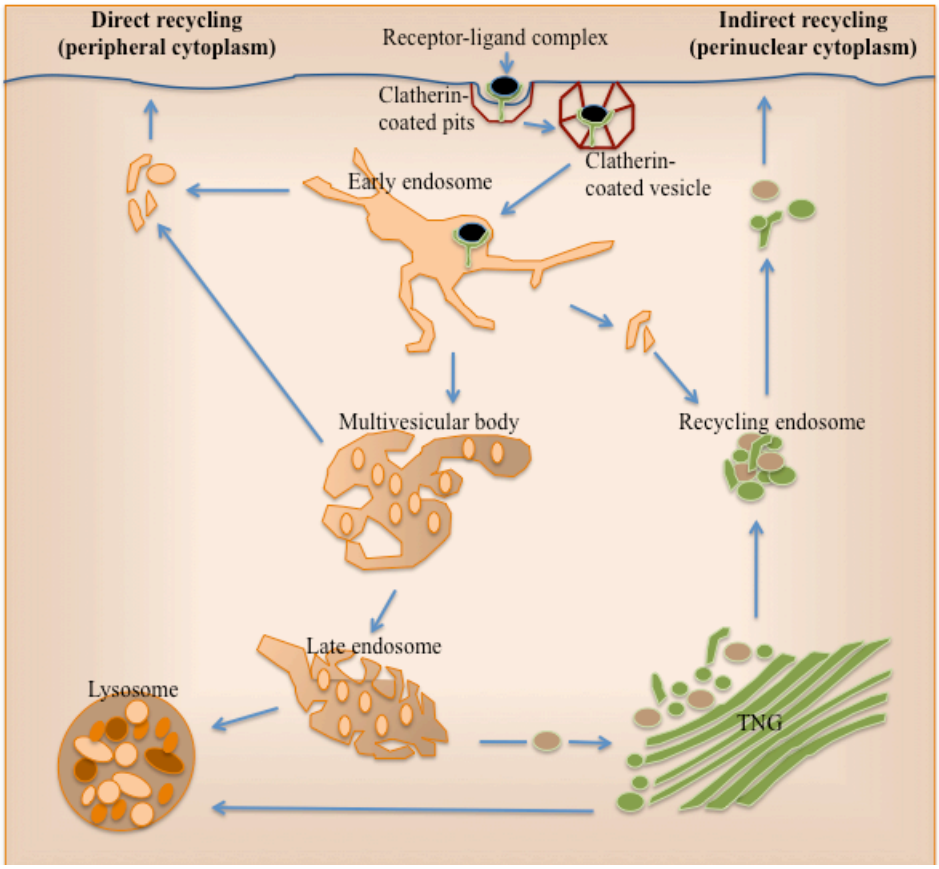


Figure 1.11. Illustration of macromolecule internalization via clatherin-mediated endocytosis followed by intracellular trafficking.

Early endosomes may also fuse into 'multivesiclular bodies' which then become 'late endosomes' having an intra-luminal membrane system. Both late endosomes and TGN components can be transported to the lysosomes, a membrane-bound cellular compartment containing nucleases, proteases and glycohydrolases which bring about macromolecular degradation. These organelles are often defined by their surface lysosomal-associated membrane proteins (LAMP) (Mukherjee et al., 1997; Conner and Schmid, 2003). Notably, most of the types of intracellular target vesicle, particularly the lysosomes, have an acidic pH that facilitates the dissociation of the receptor-ligand complexes which are then either

degraded or re-cycled back to the plasma membrane (e.g. LDL receptor, G protein-coupled receptor). A number of proteins, including ubiquitin molecules and small regulatory SMURF proteins bind to the macromolecules and direct their trafficking for lysosomal destruction (Mukherjee et al., 1997; Conner and Schmid, 2003).

Several reports suggest that at least some components present in EMD may be internalized into cells by receptor-mediated endocytosis. For example, Resland et al., (2006) reported that EMD was internalized into osteoblasts via clathrin-coated pits, observed by co-labelling the cells with anti-EMD and anti-AP-2, a clathrin adaptor protein which is responsible for linking the ligand-receptor complex (cargo) into the clathrin-coated pits intracellularly. However, in this study the binding of EMD to the cell membrane and intracellular transport and fate of EMD were not examined (Reseland et al., 2006). Moreover, EMD is a complex mixture of various proteins including amelogenin, sheathlins and enamelin (Brookes et al., 1995; Hu et al., 1997). Thus, the specificity of antibody raised against EMD and the specific component(s) internalized into the osteoblasts remain unclear in this report (Reseland et al., 2006). In addition, mouse full-length amelogenin and a spliced form, LRAP, both major components of EMP (Section 1.4.2), have been shown to be internalized and transported to epithelial cell lysosomes (Xu et al., 2008), most probably by the same clatherin-coated endocytosis. This process is crucial in cell differentiation and development as it modulates the level of surface signalling receptors and thereby the response to extracellular stimuli e.g. growth factors, cytokines, morphogens. However, at present only a little information is available about the interaction of commercially prepared heat-treated EMD/Emdogain® components with PDL cells, and, it also remains unclear how many and which type(s) of cell in the heterogeneous PDL population expresses receptor(s) capable of binding and internalization of EMD components.

1.6. Statement of problem

The precise effects of EMD and the components of EMD on the differentiation of PDL cells *in vitro* and PDL regeneration *in vivo* are still uncertain partly because:

(i) previous studies of PDL cell differentiation have been limited mainly to mesenchymal lineages (osteogenic, adipogenic and chondrogenic), while the capacity to undergo vasculogenic, angiogenic, neurogenic and gliogenic differentiation *in vitro*, the corresponding tissues being key features of PDL regeneration *in vivo*, have not been investigated *in vitro*;

(ii) there are many discrepancies and uncertainties about the effects of EMD and EMD components on bone forming cells *in vitro* and bone regeneration *in vivo*;

(iii) it is still unknown which specific EMD components may be active in regulating PDL differentiation into multiple pathways *in vitro* and modulating periodontal regeneration *in vivo.*

1.7. Hypothesis of study

It has previously been reported that the adult PDL may contain a stem cell-like population that functions as a precursor for periodontal tissue regeneration (Huang et al., 2009; Singhatanadgit et al., 2009; Menicanin et al., 2010; Kawanabe et al., 2010), although the precise characterization of these cells and their ability to differentiate into multiple lineages is not yet known. In addition, although the commercial product Emdogain® (EMD; Institut Straumann AG), derived from EMP of developing pig teeth, has been used extensively to help re-build new periodontal tissue which supports the tooth, the effects of EMD on bone regeneration have been inconclusive, possibly because EMD comprises a complex mixture of proteins that are likely to have many diverse activities on the heterogeneous cells present in the PDL. However, EMD has recently been separated into two main fractions (See section 1.4.3.2) (Mumulidu et al., 2007):

(i) Fraction C, comprising proteins < 6 kDa and containing mainly the 43 and 45-amino acid tyrosine-rich peptide (TRAP), derived from proteolytic clipping of amelogenin; and

(ii) Fraction A comprising EMD proteins > 6 KDa and containing the full-length amelogenin (18 kDa), sheathlin proteins (approximately 13-17 KDa) involved in formation of cementum (Kanazashi et al, 2006) and a 56-amino acid leucine-rich peptide (LRAP), a splicing form of amelogenin.

The hypotheses of the present study are:

(1) the heterogeneous PDL population contains precursor/progenitor/stem-like cells that have the capacity to differentiate into mature bone cells and possibly also a range of other mesenchymal and also non-mesenchymal lineages;

(2) EMD Fraction C and Fraction A and their components can differentially modulate these PDL differentiation pathways *in vitro*;

(3) amelogenin and amelogenin isoforms of EMD may exert their effects by direct cellular interaction with the PDL cell membrane followed by internalization and intracellular transport.

1.8. Aim of study

The present study was carried out to test:

1. the ability of adult PDL cells to undergo mesenchymal (osteogenic, adipogenic and chondrogenic) and non-mesenchymal (vasculogenic, angiogenic, neurogenic and gliogenic) differentiation *in vitro;*

2. the effects of EMD and the EMD Fractions on mesenchymal and non-mesenchymal differentiation of PDL cells *in vitro;*

3. the binding and intracellular fate of the EMD components in PDL cells.

Chapter 2

2.1. Isolation and maintenance of primary human PDL cells

2.1.1. Collection of PDL samples

Human PDL was obtained from the middle third of the root surface from patients undergoing routine extractions of third molar teeth at the Eastman Dental Hospital. The participants signed informed consent, in accordance with the protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital. The PDL tissue was immediately placed in low glucose-Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Life Technologies Ltd, Paisley, UK) containing 10% heat-inactivated foetal calf serum (FCS) (PAA Laboratories, Somerset, UK) (10% FCS-DMEM) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine and 25 μg/ml amphotericin B (all from Gibco).

2.1.2. Isolation of cells by enzymatic digestion of the PDL tissue

The tissue was cut into approximately 1-3 mm^3 pieces and incubated for 45 min at 37^oC in a humidified atmosphere of 5% CO_2 in air with a digestion medium of low glucose-DMEM, 3 mg/ml Collagenase type I (Sigma Chemical Co., Poole, UK), 4 mg/ml Dispase (Sigma), 2.5 μg/ml amphotericin B (Gibco) and 100 μg/ml streptomycin (Gibco). The suspension was rigorously pipetted to free any attached cells and to obtain a single cell suspension. The cells were centrifuged at 400 x g for 5 min and incubated in 6-well tissue culture plate (Nunc; Fisher Scientific, Loughborough, UK) in 3 ml of growth medium (GM), consisting of low glucose-DMEM, 2 mM L-glutamine (Gibco), 10% Fetal Calf Serum (FCS) (PAA laboratories), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin and 1% fungizone (Gibco), and cultured until the adherent PDL cells reached confluence.

2.1.3. Passage of cells

Once the cells became confluent, the medium was aspirated and the cells washed twice with PBS. The cells were then treated with trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 1 mM EDTA) (Gibco) for 5 min at 37^{o}C, followed by gentle shaking. GM was then added to neutralize the trypsin, the detached cells were collected in a Falcon tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the cell pellet re-suspended in culture medium and re-cultured in tissue culture flasks at a density of 10,000 cells/cm^2. The 'first passage' cultures were allowed to grow until they become semi-confluent, after which the cells were cryopreserved, as described in 2.1.5.

2.1.4. Cryopreservation and recovery of cells

Aliquots of the cells were cryopreserved at an early passage as a seed stock. After trypsinizing the cells, the cell pellets were re-suspended in a 'freezing medium' consisting of GM supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma). Aliquots containing approximately 2 x 10^5 cells were placed into cryotubes in a pre-freezing container (Marathon, London, UK) at -70^{o}C for 24 h, before transferring to liquid nitrogen for long-term storage. To recover the cells, the cryotubes were placed in a water bath at 37^{o}C and the thawed cell suspension rapidly added to pre-warmed GM in a sterile Falcon tube, centrifuged at 100 rpm for 5 min and cultured in 75 cm^2 tissue culture flasks as described above. In this study, three separate PDL cell populations from three different healthy donors (all male, aged between 18 to 25) were pooled together and used between passages 3 and 6. PDL cell population over passage 6 was not used in this study in order to avoid any complications resulting from possible senescence and also reduction in multi-lineage differentiation potential. In addition, heterogeneous population (but not selected/isolated homogeneous cells) of PDL cells (pooled from 3 separate patients) was used in order to examine overall response of EMD and the EMD Fractions on range of different lineage-specific precursor/progenitor/stem cells within heterogeneous PDL population.

2.1.5. Cell viability

The cultured cells were examined by phase-contrast light microscopy to assess the morphological status of the cells and any possible microbial contamination. The viability of the cells was determined at the time of each passage by trypan blue exclusion. Briefly, the cell

pellet obtained after trypsinization was re-suspended in PBS and 10 µl mixed with an equal volume of 1% trypan blue (Sigma) for 5 min. The number of cells in 10 µl of this mixture was counted under the phase-contrast light microscope, using a haemocytometer (Sigma). Viable cells appeared bright and colourless, whereas dead cells were dark blue.

2.1.6. Other cell types

Primary human AB cells were grown from cortico-lamellar bone of the maxilla of patients undergoing routine extractions at the Eastman Dental Hospital, as previously described (Singhatanadgit et al., 2008). Briefly, the bone chips were cut into approximately 1-2 mm^3 pieces, placed in 48-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 15 min to enable them to adhere to the culture plates. Low glucose- Dulbecco's Modified Eagle's Medium (Low glucose-DMEM) (Gibco) containing 10% FCS (PAA Laboratories) supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, and 2 mM L-glutamine (all from Gibco) was then added and the explants were cultured until the outgrowth of the adherent cells reached confluence.

In addition to the PDL and AB cells, bone marrow-derived mesenchymal stem cells (BMSC) (Lonza, Slough, UK) and a recently isolated 'clonogenic' cell population from adult human PDL which was shown to exhibit characteristic features of a bone progenitor/stem cell (clone 7) (Singhatanadgit et al., 2009), were also used in the present study in order to examine the effects of EMD components on a range of bone-forming cells. AB, BMSC and clone 7 were cultured in the same way as the PDL cells, described above, and used between passage 3 and 6. Human umbilical vein endothelial cells (HUVEC) were also used for testing the effects of EMD components on angiogenesis. HUVEC (Lonza) were used between passage 3 and 6 and cultured using endothelial basal medium-2 (EBM-2) (Lonza) in the same way as the PDL cells, described above.

2.2. Semi-quantitative Reverse-Transcription polymerase chain reaction (RT-PCR)

Although a number of procedures exist for measuring specific mRNA transcripts in a sample of total cellular RNA (eg, Northern blot analysis, *in situ* hybridization), the reverse-transcription polymerase chain reaction (RT-PCR) is one of the most sensitive and rapid

techniques currently available, being able to assess mRNA levels in samples as small as a single cell (Liu et al., 2004). In the present study semi-quantitative RT-PCR was used to study the regulation of lineage-associated genes of PDL cells when the cells were cultured under non-selective growth conditions and lineage-specific differentiation medium. Semi-quantitative RT-PCR was also used here to investigate the effects of EMD and the EMD Fractions on genes associated with several different lineages in order to determine whether these proteins modulate differentiation pathways of PDL cells cultured under selective differentiation conditions.

Total RNA was extracted from the cells using RNeasy® Mini Kit (Qiagen, West Sussex, UK), in accordance with the manufacturer's instructions. Briefly, 350 µl of buffer RLT with β-mercaptoethanol (10 µl in 1 ml of RLT buffer) was added on the cell monolayer to get the cell lysate. The samples were then homogenized by vigorous pipetting for 1 min. Then, 350 µl of 70% ethanol was added to the homogenized cell lysate, after which total of 700 µl of the sample, including any precipitate that may have formed, were transferred to an RNeasy mini-column placed in a 2 ml collection tube and centrifuged for 15 s at 10000 rpm. The sample was then washed with 700 µl of buffer RW1 and the column was centrifuged for 15 s at 10000 rpm. The sample was washed twice with 500 µl of buffer RPE and the column centrifuged for 2 min at 10000 rpm. Following centrifuge, the column was transferred to a new 2 ml collection tube and centrifuged for another 1 min. To elute the RNA sample, the column was then transferred to a new 1.5 ml collection tube, 25 µl of RNase-free water was added directly and the column centrifuged for 1 min at 10000 rpm. The elution step was performed twice and the total volume of 50 µl of the RNA sample stored at -70°C until further analyzed.

The initial step (i.e. RT) is the production of single-stranded complementary DNA (cDNA) copies of total RNA using a retroviral RT enzyme such as that from the Moloney Murine Leukemia Virus (MMLV). Oligo dT primers are added to hybridize with the poly-A tail of the mRNA, after which the enzyme, in the presence of the deoxynucleotide triphosphates (dNTP), extends the primer strands, resulting eventually in cDNA strands. It is then followed by exponential amplification of a specific cDNA of interest by PCR, as follows.

The PCR reaction contains the components required for DNA amplification, *i.e.* a sample of the total cDNA sequences, the four deoxynucleotides which are utilized to produce more

cDNA, the primer sequences that flank specific regions of the particular gene of interest and a DNA polymerase. The polymerase widely used is the Taq polymerase, named for *Thermus aquaticus* from which it was isolated and invaluable because of its thermostable property. The first part of PCR involves heating the product of the RT reaction at 90-95^{o}C for 30 sec in order to separate the cell-derived 'parent' mRNA strand and ensure that all of the newly-formed cDNA is in a single-strand form. The reaction is then cooled to 50-65^{o}C to enable the specific primers to 'anneal' to the ends of the target cDNA strand. The final step of the reaction is to make a complete copy of the template in the presence of the Taq polymerase, which is optimally active at 72^{o}C. The sequential addition of deoxynucleotides to the primer eventually makes a complementary copy of the original cDNA strand, completing one PCR cycle, resulting in the generation of a double-stranded DNA corresponding to the mRNA of interest. Beginning again with heating at 90-95^{o}C to separate the two cDNA strands, this protocol can be repeated 30 or more times, each newly-synthesized cDNA sequence acting as a new template at each cycle. Theoretically, the amplification for a given number of cycles can be calculated using the formula:

$$Y = A \times 2^{n} \text{ where A: initial number of copies; n = number of cycles}$$

The initial cycles of PCR are characterized by an exponential increase in target amplification in the presence of an excess of reactants and when the enzyme is fully active. However, when these conditions are no longer present the product stops accumulating exponentially and a plateau is reached at which there is little or no net increase in PCR product. Thus, for semi-quantitation, the PCR reaction is terminated when the products from both an internal 'control' gene sequence and a gene of interest are detectable and are being amplified within a linear range. Since it is usually possible to ensure that these conditions are met, RT-PCR is a rapid, inexpensive, simple and sensitive method for detecting and accessing the relative level of expression of specific genes.

In the present study RT-PCR was therefore used to monitor the effects of EMD, the EMD fractions and components on the ability of the PDL cells to express genes associated with different cell phenotypes when cultured in non-selective medium. The PDL cells were incubated in growth medium for 4-5 days until 90% confluence, then cultured in the presence of the proteins for another 7 days. Total RNA was extracted from the cells using RNeasy Mini

Kit, in accordance with the manufacturer's instructions. For the RT reaction, 5 µg of total RNA was used with 5 ng of oligo-dT (Promega; Madison, WI) in 40 µl of water. After 5 min at 65°C, the first stand of cDNA was synthesized in a total volume of 50 µl, containing 50 U of M-MuLV RT, 1x M-MuLV buffer, 40 µM of each dNTP and 40 U of RNase block (all Stratagene; La Jolla, CA) to stop RNase activity. After incubation at 37°C for 60 min, the RT enzyme was inactivated by incubation for 5 min at 90°C. 10 µl Of each cDNA sample was then added to a 50 µl reaction mix containing 2 U RED-Taq DNA polymerase, 1x RED-Taq PCR buffer, 50 µM of each dNTP (all Sigma) and 2 µM of the respective sense and antisense primer pair sequences (Sigma-Genosys, Pampisford, UK). The sequences of the primers for the lineage-specific genes are shown in Table 1.

An initial denaturation step of 2 min at 94°C was followed by amplification cycles (30 sec at 94°C, 30 sec at the gene-specific annealing temperature (Table 2) and 30 s at 72°C) and final extension for 7 min at 72°C. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified to assess the quality of the extracted RNA (Das and Olsen, 2001) and as the baseline gene for comparing the target genes of interest. The PCR products were separated by agarose gel electrophoresis on a 2% agarose gel, consisting of 2% agarose in 1x TAE buffer (40 mM Tris base, 40 mM acetic acid and 1 mM EDTA, pH), with 0.5% µg/ml of ethidium bromide (all from Sigma), a fluorescent dye that intercalates between the nucleic acid bases and enables visulization of the PCR DNA product in the gel under UV light. The electrophoresis was carried out using 1x TAE buffer, with the voltage applied at 5 volts per cm of the distance between the two electrodes of the electrophoresis tank. To obtain a semi-quantitative estimate of PDL gene mRNA levels, the intensities of the bands corresponding to the target genes were measured using the ImageJ software for analysis (ImageJ, NIH, USA) and compared with that of the GAPDH gene intensity, defined as 1.0.

Table 2. Semi-quantitative RT-PCR primers of selected lineage-specific genes

Lineages	Gene	Primers*	Annealing temperature (^{o}C)	Cycles	Product size (bp)
Osteogenic	Runx2 (early)	(F) CAGTTCCCAAGCATTTCATCC (R) TCAATATGGTCGCCAAACAG	55	30	159
	BSP (late)	(F) TGCTCAGCATTTTGGGAAT (R) TGCATTGGCTCCAGTGACACT	58	30	627
Adipogenic	PPARγ2 (early)	(F) TCTGGCCTATGGACTCAGCT (R) GCAGAGGACGTCTCTATGCC	55	30	172
	LPL (late)	(F) TCAAGGATGTTCTGGCTTTAC (R) GAGTTGGCTCTGTGAGACC	58	30	167
Chondrogenic	Sox-9 (early)	(F) AGTACCCGCATCTGCACAAC (R) TACTTGTAATCGGGGTGGTCT	58	35	631
	Aggrecan (late)	(F) CTGCAACTGAAGTGCCC (R) AGGCTGATGGTTCCTCTGA	57	35	186
	Col2a1 (late)	(F) AAACAACACAATCCGTTGCA (R) TTGTGTAGGACACACACAGTTCC	57	30	345
Myogenic	MyoD1 (early)	(F) CCTTTGCCACAACGGACGACTTCT (R) TGCGAGTGCTCTTCGGGTTTCAG	60	30	127
	Desmin (late)	(F) CAGGGTGGACTTAGAAAGCA (R) ATTGTTTCTCTCCAGAGCCC	55	35	210
Vasculogenic	Flt-1/VEGFR1 (early)	(F) CTGACCCTTCTACATTTGAGGG (R) ACTAGAGGAGCCAAAAGTATGGG	60	35	231
	Flk-1/VEGFR2 (early)	(F) TGCACTGCAGACAGATCTAC (R) GCAGACATAGTCTCCTTGGT	60	35	215
	Tie-1 (late)	(F) GCCATGATCAAGAAGGGACGG (R) GTTCTCTCCGACCAGCACAT	60	35	404
	Tie-2 (late)	(F) CCTTCCTACCTGCTACTTTA (R) CCACTACACCTTTCTTTACA	60	35	434
	VE-cadherin (late)	(F) CTGGGCACCGACTCATCCGACTCT (R) GGCCCCTTCCTCCCTCTTGA AG	60	35	715
Neurogenic	NGF (early)	(F) GATGCCAGATTAGGGATCTGCTGG (R) ACTCCTGCTTCTGGCAGCTGCAG	55	30	497
	MAP-2 (late)	(F) CCATTTGCAACAGGAAGACAC (R) CAGCTCAAATGCTTTGCAACTAT	60	35	135
	PGP9.5 (late)	(F) GTCTTGCTCTGTCACCCAGG (R) GATCAGGAGTTCCAGACCAGC	58	35	204
	NF-L (late)	(F) GCAGTAGTGCCGCAGTTTCA (R) TGCAATTCATCTTCCTTTCT	58	35	138
Gliogenic	Notch1 (early)	(F) CACCATCAGTATCATTTTTATTGC (R) CACTCCTCAGGGCAGCCT	58	30	227
	GFAP (late)	(F) GTGCCCTACTTCCATCCTGA (R) TGTGGACAAATCTGTTACAGCC	61	35	126

*** (F) Forward; (R) Reverse**

2.3. Quantitative real-time PCR (Q-PCR)

Q-PCR was used to study the effects of EMD and the EMD Fractions on osteogenic genes Runx-2, osteopontin (OP), osteocalcin (OC) and bone sialoprotein (BSP) of PDL cells cultured in osteogenic media.

The difference between the Q-PCR and semi-quantitative RT-PCR is that Q-PCR amplifies and simultaneously quantifies a target DNA molecule as it is produced directly at each PCR cycle. This detection and quantification of the number of copies is then normalized to that of an 'internal' control DNA such as GAPDH as it is produced. This generates a rate of cDNA formation which is equivalent to the amount of cDNA present initially whereas semi-quantitative RT-PCR measures the relative level of cDNA product only at one particular time of exponential amplification, and may be affected by reagents, non-specific binding signals etc. In addition, Q-PCR employs a much more sensitive detection system than ethidium bromide staining of gel used in RT-PCR.

Two systems are generally used for Q-PCR: The SYBR Green I Dye-based system employs the SYBR Green I dye which binds to the double-stranded (ds) DNA product, resulting in an increase in fluorescence intensity which is proportional to the amount of product at each time examined and thus the rate of ds-PCR product formation. The disadvantage of this system is that the dye also binds to non-specific ds-DNA sequences, resulting in false positive signals. The Taqman probe-based system uses a fluorogenic probe which detects the specific PCR product as it accumulates during the PCR cycle, thus combining specificity of the primers and the probes and generating highly specific signals which are directly proportional to the initial level of the target cDNA. This Taqman Probe-based Q-PCR system was used in present study.

For quantification of the lineage-specific genes by RT-Q-PCR, PDL cells were cultured in osteogenic medium (OM) in the presence of EMD and the EMD Fractions. Total RNA was isolated and primers specific for Runx-2, Osteopontin, Osteocalcin, bone sialoprotein and GAPDH genes were used for Q-PCR (Primer sequences were obtained from Applied Biosystems, Foster City, CA, USA). For Q-PCR analysis, the ABI Prism® 7300 sequences detector (Applied Biosystems), the Taqman® Gene Expression Assay consisting of unlabelled specific PCR primers and Taqman® MGB probes with FAM™ dye-labelling were used. The PCR reactions were carried out in a 96-well plate format as recommended by the manufacturer. Briefly, the cDNA products were first diluted (1:2) in nuclease-free water and a

5 µl aliquot was used in a 25 µl reaction mixture containing 12.5 µl of 2x Taqman® Universal Assay mix (P/N 4304437), 1.25 µl of gene expression assay mix specific for each gene listed in Table 2 (all from Applied Biosystems) and 6.25 µl of nuclease-free water. Thermal cycler conditions were used as recommended by the manufacturer, which consisted of AmpErase® UNG activation at 50°C for 2 min, AmpliTaq Gold® DNA polymerase activation at 95°C for 10 min and 40 PCR cycles, each of which was 95°C for 15 s and 60°C for 60 s. The data were collected and analyzed by the SDS 1.2 software (Applied Biosystems).

All PCR reactions were performed in triplicate and each of the gene signals were normalized to the GAPDH signal detected simultaneously on the same plate. The data are presented as the mean fold-induction (± SE) compared with that of control cells cultured in osteogenic media in the absence of any protein additions (defined as 1.0).

2.4. Flow cytometry (FCM)

FCM is commonly used technique to measure certain physical and chemical features of individual cells in a cell suspension as it passes through a fixed laser beam and is based on the angular reflection of an incident laser (Carter and Ormerod, 2000; Shapiro, 2003). Light reflected by the cells at low angle (< 2°) is detected in the forward direction along the axis of the incident light and this "forward scatter" (FSC) is considered to be proportional to the relative size of the cell (Carter and Ormerod, 2000). Light reflected at more than < 2°is detected at 90°C or more to the light axis and is referred to as orthogonal or "side scatter" (SSC), which is considered to be proportional to the cytoplasmic granularity or internal complexity of the cell (Carter and Ormerod, 2000).

Using this procedure, the relative levels of cell-associated proteins can also be measured, by first treating the cells with a fluorochrome 'tag' or fluorescent-labelled tag or antibody which is specific for the target antigen (Carter and Ormerod, 2000). When laser light is applied to the cell, the fluorochrome absorbs energy from the laser and subsequently releases this energy by emitting fluorescence at a particular wavelength. The intensity of this emitted fluorescence is proportional to the amount of fluorochrome-labelled antibody, which is in turn proportional to the level of antigen. A FACScan flow cytometer (Becton Dickinson) employing an argon-ion laser for excitation was used for FCM in this study. The electronic system of the instrument was optimized for collection of the data and the same instrument

settings were used to perform all experiments. Moreover, by the use of appropriate concentration of a specific non-ionic detergent (such as Triton X) which permeabilised the cell membrane and so enables the entry of high molecular weight 'detecting' antibodies, it was possible to measure the relative levels of intracellular as well as cell surface antigen, as previously described (Kuru et al., 1998). Detailed protocols for preparing the cells for FCM are discussed in **Chapters 4 and 6**.

2.5. Immunogold electron microscopy (EM)

Immunogold EM is a technique that utilizes a transmission EM (TEM) to elucidate the precise ultrastructural localization of antigens in cells or tissue samples. This can be carried out by a process known as immunogold labeling, in which careful sample preparation is essential for preserving both the integrity of the sample and antigenicity of the protein to be detected. Sections are incubated with specific antibodies against the antigen of interest. Since these detecting antibodies are not visible in the TEM, their presence (i.e., their binding to the corresponding antigen) is made electron-dense and thus EM-visible by incubating with a second antibody conjugated to a small gold particle, which is electron-dense. The sections are then stained with uranyl acetate and lead citrate to enhance the contrast between intracellular structures of differing electron density. Despite the labour-intesive effort required for this work, imnmunogold EM has a significant number of advantages over light microscopic procedures for investigating specific subcellular structures and the precise location of antigens of interest.

2.6. Statistical analysis

The data are presented as the mean ± SE of measurements of three separate experiments. Statistical differences between the means were analyzed by one-way ANOVA, followed by the *post-hoc* Bonferroni test for multiple comparisons ($p<0.05$), unless otherwise stated in the result chapters (SPSS 11.0 software, Chicago, IL).

Chapter 3

3.1. Introduction

Stem cells are generally characterized by their ability to give rise to at least one differentiated cell type as well as having the unique capacity to self-renew (Collins et al., 2005). In contrast to the large majority of the cell populations of adult tissues that are committed to a specific function, stem cells are uncommitted until they receive signals to differentiate into progenitor/precursor cells before forming fully differentiated and functionally active cells (Lemoli et al., 2005). The presence of such cells is considered to be critical for tissue homeostasis and the replenishment of cells that have died because of apoptosis, injury and disease (Weissman et al., 2000; Till and McCulloch, 1961).

Stem cell-like populations have now been identified in a number of adult human tissues, including the central nervous system (CNS), olfactory and dermal epithelium, blood vessels, skeletal muscle and adipose tissue (Johe et al., 1996; Barnett et al., 2004; Cotsarelis et al., 1990; Neimann et al., 2002; Asahara et al., 1997; Collins et al., 2005). While PDL cells have been shown to be able to differentiate along mesenchymal lineages (osteogenic, adipogenic and chondrogenic), their 'non-mesenchymal' vasculogenic, angiogenic, neurogenic and gliogenic lineage-associated differentiation capability is not known, despite the physiological and structural importance of these lineages in the PDL (Seo et al., 2004; Singhatanadgit et al., 2009, Xu et al., 2009). Thus, although identification, isolation and characterization of PDL stem cells with multi-lineage differentiation capacity is clearly highly relevant to periodontal regeneration, their presence within the PDL remains unclear due to lack of definitive phenotypic signatures and biochemical features. The present study has therefore examined the multi-lineage differentiation potential of adult human PDL cells using the conventional reverse transcription polymerase chain reaction (RT-PCR) technique to assess the expression of selected 'key' marker genes that are associated with mesenchymal and non-mesenchymal pathways of stem cell differentiation and, secondly, by using standard histological, immunological and morphological criteria to assess characteristic features of lineage-specific differentiation *in vitro*.

3.2. Materials and Methods

PDL cell culture and RT-PCR were performed as described in Chapter 2; additional methods used in this chapter are described below.

3.2.1. Analysis of lineage-associated genes using semi-quantitative RT-PCR

Conventional RT-PCR was used to assess the expression of lineage-associated genes as described in Chapter 2 and in a previous report (Singhatanadgit et al., 2009). This was carried out by first incubating the cells in a standard non-selective growth medium (GM) to determine the 'baseline' expression of the selected genes. The cells were then cultured in specialized media previously shown to support the differentiation of precursor cells into specific lineages (differentiation medium; DM). The following genes were analyzed as 'key' markers of the different lineages which have previously been reported to be expressed by precursor cells at 'early' or 'late' stages of their differentiation into specific mature functional cells: the osteogenesis-associated marker genes Runt-related transcription factor 2 (Runx2) (early) and bone sialoprotein (BSP) (late); the adipogenesis-associated genes peroxisome proliferator activator receptor γ2 (PPARγ2) (early) and lipoprotein lipase (LPL) (late); the chondrogenesis-associated genes Sox-9 (early) and aggrecan (late); the myogenesis-associated genes MyoD1 (early) and desmin (late); the vasculogenesis-associated genes vascular endothelial growth factor receptor 2 (Flk-1) (early) and vascular endothelial cadherin (VE-cadherin) (late); the neuronal differentiation marker genes nerve growth factor (NGF) (early) and microtubule-associated protein (MAP2) (late); the glial-associated Notch1 (early), glial fibril acidic protein (GFAP) (late) and myelinated oligodendrocyte basic protein (MOBP) (late) genes; and a house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequences of the forward and reverse primers (from Sigma-Genosys, Pampisford, UK), annealing temperature, number of amplification cycles and expected product sizes (bp) are shown in Chapter 2. To obtain a semi-quantitative estimate of the relative level of mRNA transcript expression, the cDNA intensity of the band corresponding to each PCR product was measured by densitometry using the ImageJ (NIH software) and normalized to that of GAPDH used as an internal control.

3.2.2. Analysis of characteristic features of cell differentiation *in vitro*

In these experiments the cells were cultured in various differentiation media (DM) that facilitate the *in vitro* development of 'mature' cells from putative precursor/stem cells.

3.2.2.1 Mesenchymal differentiation pathways

i. Osteogenic differentiation

PDL cells were seeded into 24-well plates at a density of 2.5 x 10^4 cells/well and cultured in GM for 3 days, then osteogenic medium (OM) added, consisting of GM supplemented with 0.1 mM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate and 10 nM dexamethasone (all from Sigma). The GM and OM were changed every 3-4 days. On days 7 and 14, total RNA was extracted and RT-PCR carried out on day 7 to measure the early marker gene Runx2 and on day 14 to measure the late marker gene BSP, as previously described (Zhou et al., 2008).

The formation of mineralized nodules, a key functional aspect of terminal osteogenic differentiation, was determined by alizarin red S staining of calcium-containing deposits, as follows. Cells were cultured in GM and OM for 21 days, fixed with 10% formaldehyde for 15 min and washed with distilled water. The samples were incubated with 2% Alizarin Red S (pH 4.2) (Sigma) for 15 min at room temperature, then washed with ice cold methanol, air-dried and photographed. The level of alizarin red staining was quantified by absorbance at 562 nm (A_{562}), after eluting the stain for 2 h with 10% cetylpyridinium chloride in 10 nM sodium phosphate buffer, pH 7.0.

ii. Adipogenic differentiation

PDL cells were cultured in GM as above, then adipogenic medium (AM) added, consisting of GM supplemented with 10 nM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine and 50 μM indomethacine (all from Sigma). The GM and AM were changed every 3-4 days. After culture in AM for 3 and 5 weeks, total RNA was extracted in order to measure the early adipogenic marker gene PPARγ2 (week 3) and the late marker gene LPL (at week 5), as described previously (Zhou et al., 2008).

Oil red O staining was carried out after 5 weeks of culture in GM or AM to assess terminal adipogenic differentiation, as follows. The cells were fixed with 10% formalin for 30 min, incubated with 60% propan-2-ol for 5 min and then stained with 0.2% Oil Red O in

propan-2-ol for 5 min. After washing with tap water, the samples were counterstained with Harris Hematoxylin. For quantification of Oil Red O, the samples (without counter staining) were extracted into 100% propan-2-ol and the absorbance measured at 490 nm (A_{490}).

iii. Chondrogenic differentiation

Cells were trypsinized and $2.5x10^5$ cells suspended in 0.5 ml of serum-free chondrogenic medium (CM) containing low glucose-DMEM, 100 ng/ml transforming growth factor-β3 (TGF-β3), 100 nM dexamethasone, 0.1 mM L-ascorbate2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline and 6.25/6.25/6.25 μg/ml insulin/transferrin/selenium and placed in 15 ml conical polypropylene centrifuge tubes (Nunc). After centrifugation at 200g for 5 min, the pellets were incubated at 37°C for 2 weeks, with caps loosened to allow gas exchange, then total RNA extracted for RT-PCR analysis of the early chondrogenic marker gene Sox-9 (after 1 week) and the late marker aggrecan (after 2 weeks), as previously described (Narukawa et al., 2006).

Alcian blue staining for acid mucopolysaccharides and glycosaminoglycans was also carried out, after 3 weeks, to assess terminal chondrogenic differentiation as follows. Cell pellets were fixed in 10% formalin at 4˚C for 24 h, dehydrated in an ascending series of ethanol (50 to 100%) and embedded in paraffin. Sections (3 μm) were cut, stained with 1% Alcian blue (pH 2.5) (Sigma) for 5 min. The deposition of mucopolysaccharide and glycosaminoglycan was visualized as blue staining of the extracellular matrix (ECM). Nuclei were stained purple using Harris Hematoxylin.

iv. Myogenic differentiation

PDL cells were cultured on 1% gelatin (Sigma) coated 24-well tissue culture plates in GM for 2-3 days, then myogenic medium (MM) added, consisting of high glucose-DMEM supplemented with 2% horse serum (PAA laboratories), 2% chick embryo extract (EggTech, Salisbury, UK) and 100 ng/ml IGF-1 (Sigma). The GM and MM were changed every 3-4 days. After culture in MM for 3 and 5 weeks, total RNA was extracted in order to measure the early myogenic marker MyoD1 (week 3) and the late marker desmin (at week 5), as described previously (Sinanan et al., 2004).

Immunostaining for desmin (after 5 weeks) was also carried out, as previously described (Singhatanadgit et al., 2008). Briefly, cells were fixed with 4% paraformaldehyde (Merck, Poole, UK) for 15 min at room temperature (RT) and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10%

normal goat serum (NGS) in PBS for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti-desmin antibody (Sigma) diluted 1:500 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. The desmin-positive myotube-like structures were visualized as green fluorescent stained cells. Nuclei were stained blue using Hoechst dye. The proportion of desmin-positive cells was determined by manual counting of 5 separate fields of each culture.

3.2.2.2. Non-mesenchymal differentiation pathways

i. Vasculogenic and angiogenic differentiation

In order to assess the potential of PDL cells to undergo vasculogenesis, a process by which stem cells differentiate into endothelial precursors (Damir et al., 2007), and angiogenesis, a process by which endothelial cells form morphologically distinct tubular blood vessel-like networks (Damir et al., 2007), the PDL cells were cultured in GM as described above and then endothelial basal medium-2 (Lonza, Wokingham, UK) (EBM-2) was then added, consisting of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Total RNA was extracted to measure the early vasculogenesis-associated marker gene (Flk-1; at week 2) and a late marker gene (VE-cadherin; at week 5) associated with this process, as described previously (Lee et al., 2009).

Further assessment of terminal vasculogenic differentiation was also carried out by immunostaining for VE-cadherin (after 5 weeks), as previously described (Singhatanadgit et al., 2008). Briefly, cells were fixed with 4% paraformaldehyde (Merck, Poole, UK) for 15 min at room temperature (RT) and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% NGS in PBS for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti-VE-cadherin antibody (Insight Biotechnology, Wembley, UK) diluted 1:100 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. The vasculogenic VE-cadherin-positive endothelial cell-like population was visualized as green fluorescent stained cells. Nuclei were stained blue using Hoechst dye. The proportion of VE-cadherin-positive cells was determined by manual counting of 5 separate fields of each culture.

Angiogenic differentiation of PDL cells was performed using an *in vitro* angiogenesis assay kit (Millipore, Billerica, MA, USA). Briefly, 10^4 cells were plated on ECMatrix gel

coated 96-well plates and cultured in the presence of EBM-2. After 6 h, digital images were obtained using bright-field microscopy to visualize morphological evidence of angiogenic structures, which were scored from 0 to 5, as previously described (Schlueter et al., 2007), based on the progressive appearance of morphological features associated with angiogenic structures: individual cells, (0); aligned cells, (1); capillary tubes without sprouting, (2); sprouting of new capillaries, (3); the formation of closed polygonal structures, (4); and complex-mesh-like structures, (5). Each condition for each culture was scored using 5 separate fields.

ii. Neural differentiation

In order to assess the ability of PDL cells to undergo neurogenesis, the process by which nerve cells are generated from stem cells/neuronal precursors, 2 x 10^4 cells/well were cultured in poly-L-lysine/laminin coated 8-well chamber slides (Nunc) in GM for 2 days and neural differentiation medium (NM) then added, consisting of 20 ng/ml EGF, 50 ng/ml bFGF, 4 μM forskolin, 10 μM retinoic acid, 50 ng/ml brain derived neurotrophic factor (BDNF) and 50 ng/ml neurotrophin-3 (NT-3) (all from Sigma). Total RNA was extracted and the early neuronal marker gene NGF (day 2) and late gene MAP-2 (day 7) were measured, as previously described (Coura et al., 2008).

Immunostaining for neuron-specific βIII tubulin was carried out as previously described (Singhatanadgit et al., 2009). Briefly, cells were fixed with 4% paraformaldehyde (Merck, Poole, UK) for 15 min at RT and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% NGS in PBS for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti-βIII tubulin diluted 1:1000 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. Neuron-like cells were visualized by their green fluorescent staining and long axonal projections. Nuclei were stained blue using Hoechst dye. The proportion of neuron-specific βIII tubulin-positive cells was determined by manual counting of 5 separate fields of each culture.

iii. Glial differentiation

The capacity of PDL cells to undergo glial differentiation, the process by which three distinct types of myelinating cells (astrocytes and oligodendrocytes found in the central nervous system and Schwann cells found in the peripheral nervous system (PNS)) are generated, 2 x 10^4 cells/well were cultured in 8-well chamber slides (Nunc) in GM for 2 days and glial differentiation medium (GDM) then added, consisting of DMEM/F-12, 2% FCS and 4 μM forskolin in order to facilitate astrocytic differentiation. The GDM was further supplemented with 10 ng/ml heregulinβ2 to enable oligodendrocyte and Schwann cell differentiation. Total RNA was extracted and Notch1 (day 4) (a common marker of all three gliogenic lineages), GFAP (day 10) (a late marker specific for the astrocytic lineage) and MOBP (day 10) (an oligodendrocytic and Schwann cell-associated marker gene) were measured as previously described (Coura et al., 2008).

Immunostaining was carried out for GFAP and O4, markers of astrocytes and of oligodendrocytes and Schwann cells, respectively, as previously described[18]. Briefly, cells were fixed with 4% paraformaldehyde (Merck, Poole, UK) for 15 min at RT and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% NGS in PBS for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti-GFAP (R&D Systems, Minneapolis, MN, USA) and anti-oligodendrocyte O4 (R&D Systems, Minneapolis, MN, USA) diluted 1:500 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. Astrocyte-like cells were visualized by their red fluorescent staining and 3-10 long dendritic projections, and oligodendrocyte-like cells were visualized by their yellow fluorescent staining and 20-30 dendritic projections. Schwann-like cells were visualized by the yellow fluorescent staining of their small cell body (soma) and two myelinating dendrites on opposite sides of the soma. Nuclei were stained blue using Hoechst dye. The proportions of astrocyte-specific GFAP-positive cells and of oligodendrocyte and Schwann cell-associated O4-positive cells were determined by manual counting of 5 separate fields of each culture.

3.2.3. Statistical analysis

The RT-PCR data are presented as the mean of the triplicate measurements of cells cultured in DM compared with that of control cells cultured in GM (defined as 1.0). Student's paired T test was used for statistical comparison of the test and control conditions (SPSS 12.0 software, Chicago, IL).

3.3. Results

3.3.1. Mesenchymal lineage-specification of PDL cells

The expression of mesenchymal lineage-associated genes of PDL cells cultured in non-differentiation-inducing GM was examined. Figure 3.1 shows that the PDL cells cultured in GM constitutively expressed the bone-associated genes Runx2 and BSP, the adipogenic genes PPARγ2 and LPL, the chondrogenic genes Sox-9 and Aggrecan and the myogenic genes MyoD1 and desmin, suggesting that even when incubated in medium that was not optimized to elicit any specific pathway of 'terminal' cell differentiation *in vitro*, at least some cells in the PDL cell population expressed basal levels of genes associated with a number of different mesenchymal lineages.

The differentiation potential of PDL cells into mesenchymal lineages when cultured in various differentiation media (DM) that facilitate the *in vitro* development of 'mature' cells from putative precursor/stem cells was examined. Thus, in OM, which facilitates osteogenesis, the early osteogenic transcription factor Runx2 and the late osteogenic gene BSP were found to be significantly up-regulated (1.4- and 2.2-fold, respectively; $p<0.05$) compared with cells cultured in GM alone. When cultured in adipogenesis-inducing AM, there was a 3.3-fold increase in expression of the early transcription factor PPARγ2 gene and a 4.9-fold increase in the late adipogenic gene LPL compared with GM alone, as shown in Figure 3.1 (B) ($p<0.05$). After culture in CM, the early chondrogenic gene Sox-9 and the late chondrogenic gene Aggrecan were significantly up-regulated (3.2- and 1.7-fold, respectively; $p<0.05$). When PDL cells cultured under myogenesis-inducing MM, although no significant change in the early myogenic gene myoD1 was observed (1.2-fold), the late gene desmin was significantly up-regulated (1.6-fold increase; $p<0.05$) compared with GM alone. These results thus demonstrate that at least some cells present in the adult PDL are capable of expressing significantly elevated levels of genes that delineate terminal differentiation to several distinct mesenchymal lineages (osteogenic, adipogenic, chondrogenic, myogenic). When the PDL cells were cultured in GM alone, there was little evidence of any terminal differentiation to mesenchymal lineages, on the basis of cell function and morphology *in vitro* as described in the Section 3.2 (Fig. 3.1). However, when cultured in osteogenesis-inducing OM, the cells formed higher amounts of distinct bone-like nodules compared with cells cultured under baseline conditions (in GM), exhibiting significantly higher alizarin red staining intensity (1.14 compared with 0.57; $p<0.05$) (Fig. 3.1 (A)). Similarly, when cultured in adipogenic

AM, the PDL cells produced significantly greater amounts of oil red O-positive lipid-like droplets, a functional characteristic of mature adipocytes (staining intensity of 1.32 compared with 0.50 in GM alone; $p<0.05$) (Fig. 3.1 (B)). In CM, the PDL cell pellet cultures were found to exhibit higher levels of Alcian blue staining of the ECM, indicating greater amounts of acid mucopolysaccharides and glycosaminoglycans characteristic of chondrogenic differentiation *in vitro* compared with GM (Fig. 3.1 (C)), and in MM the PDL cells produced 11.9% desmin-positive myotube-like structures compared with cells cultured in the baseline non-selective GM (0.0%). However, no multinucleated myotubes (containing three or more nuclei), a characteristic feature of terminally differentiated muscle cells, were detected (Fig. 3.1 (D)) (Collins et al., 2005).

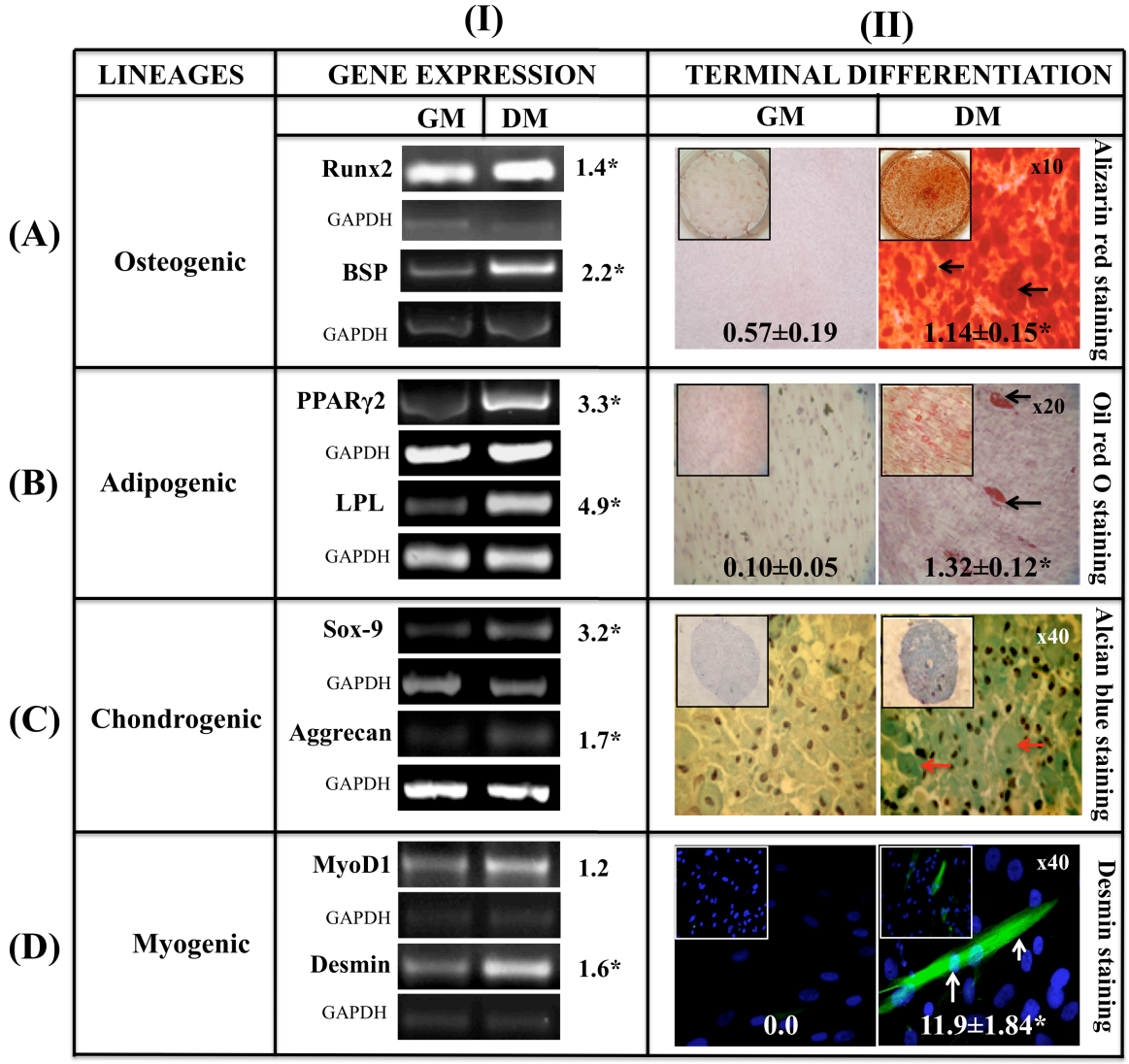


Figure 3.1. Differentiation of PDL cells to mesenchymal lineages in vitro.

(I) Representative RT-PCR gels of the expression of lineage-associated genes by PDL cells cultured in non-selective GM and in lineage-specific differentiation media (DM). The values are the changes in PCR product band intensity (relative to GAPDH) obtained in DM compared with GM, defined as 1.0;

(II) Terminal differentiation characteristics. (A) Osteogenic differentiation: Alizarin red staining of PDL cells cultured for 3 weeks in GM and in DM. The black arrows show the alizarin red positive bone-like nodules, and the numbers are the alizarin red staining intensities. Magnification x10; (B) Adipogenic differentiation: Oil red O staining of the PDL cells cultured for 5 weeks in GM and in DM. Nuclei are counter-stained purple with Harris Hematoxylin, and the back arrows show lipid-like droplets stained red with Oil red O, and the numbers are the Oil red O staining intensities. Magnification x20; (C) Chondrogenic

*differentiation: Alcian blue staining of paraffin sections of pellet cultures maintained for 3 weeks in GM and in CM. Nuclei are counter-stained purple with Harris Hematoxylin, and the red arrows show the ECM stained with Alcian blue. Magnification x40; (D) Myogenic differentiation: Immunostaining of desmin after 5 weeks of culture in GM and in MM. The numbers are the % of desmin-positive cells. Nuclei are stained blue with Hoechst dye, and the white arrows show the green fluorescence-stained myotube-like structures that were devoid of multiple nuclei. Magnification x40. The values are the means ±SE of triplicate measurements. *Indicates statistically significant difference compared with GM alone ($p<0.05$).*

3.3.2. Non-mesenchymal lineage-specification of PDL cells

The expression of non-mesenchymal pathways of vasculogenesis/angiogenesis, neurogenesis and gliogenesis-associated genes was first examined when the PDL cells were cultured in non-selective GM. The results in Figures 3.2 and 3.3 show that the PDL cells constitutively expressed genes associated with all of these lineages. Thus, the vasculogenesis/angiogenesis-associated gene Flk-1, the neurogenesis-associated genes NGF and MAP-2 and the gliogenesis-associated genes Notch1, GFAP and MOBP were constitutively expressed by the PDL cells cultured in non-differentiation-inducing GM (Figs. 3.2 and 3.3). However, lineage-specific gene expression was nevertheless selective, as the late-vasculogenic/angiogenic marker VE-cadherin was not detected.

The vasculogenic, neurogenic and gliogenic differentiation potential was also examined after the PDL cells were cultured in lineage-specific differentiation media (DM). Thus, PDL cells cultured in vasculogenic/angiogenic differentiation media (EBM-2) appeared to exhibit elevated levels of vasculogenesis-associated early gene Flk-1 and late gene VE-cadherin expression compared with PDL cells cultured in GM (1.6- and 15.5-fold increase; $p<0.05$) (Fig. 3.2). Similarly, after culture in NM, neuronal genes NGF and MAP-2 were significantly up-regulated compared with GM (2.2 and 1.8-fold increase; $p<0.05$) (Fig. 3.3). The astroglial genes Notch1 and GFAP were found to be markedly stimulated when PDL cells cultured in GDM (1.9 and 2.8-fold increase; $p<0.05$). Moreover, when GDM was supplemented with heregulinβ2, the early oligodendrocytic gene Notch1 and the late MOBP were both up-regulated compared with cells cultured in GM alone (2.0- and 3.3-fold increase, respectively; $p<0.05$) (Fig. 3.3). These results thus demonstrate that, under the appropriate conditions, PDL cells can express significantly elevated levels of vasculogenic/angiogenic, neurogenic and gliogenic genes.

Analysis of vasculogenic differentiation showed that, in EBM-2, 8.5% of the PDL cells were VE-cadherin-positive, morphologically appearing as blood vessel-like structures as shown in Figure 3.2 (A). Furthermore, cells cultured on an ECMatrix substrate in EBM-2 formed a tubular network, morphologically characteristic of angiogenesis *in vitro*, and had an angiogenic score of 2.6, while PDL cells cultured on the ECMatrix in the baseline GM alone were individual cells without any apparent tubule formation (mean score of 0.0) (Fig. 3 (ii); $p<0.05$).

Examination of the neurogenic pathway showed that, when cultured in NM, the PDL cells formed significantly more neuron-specific βIII tubulin-positive neuron-like structures exhibiting long axonal projections and neuronal dendrites, compared with GM (14.9%

compared with 0.0%; $p<0.05$) (Fig. 3.3 (A)). When cultured in GDM, 36.2% of the cells expressed the astrocyte-associated marker protein GFAP and exhibited astrocyte-like morphology (Fig. 3.3 (B)), similar to those previously observed (Chi et al., 2010). Moreover, when the GDM was supplemented with heregulinβ2, 7.4% of PDL cells expressed the oligodendrocyte and Schwann cell-specific marker O4 and exhibited oligodendrocyte-like morphology with 20-30 dendritic projections and Schwann cell-like morphology as evidenced by a small cell body with two myelinating dendrites (Fig. 3.3 (C); $p<0.05$), similar to those previously observed (Chi et al., 2010).

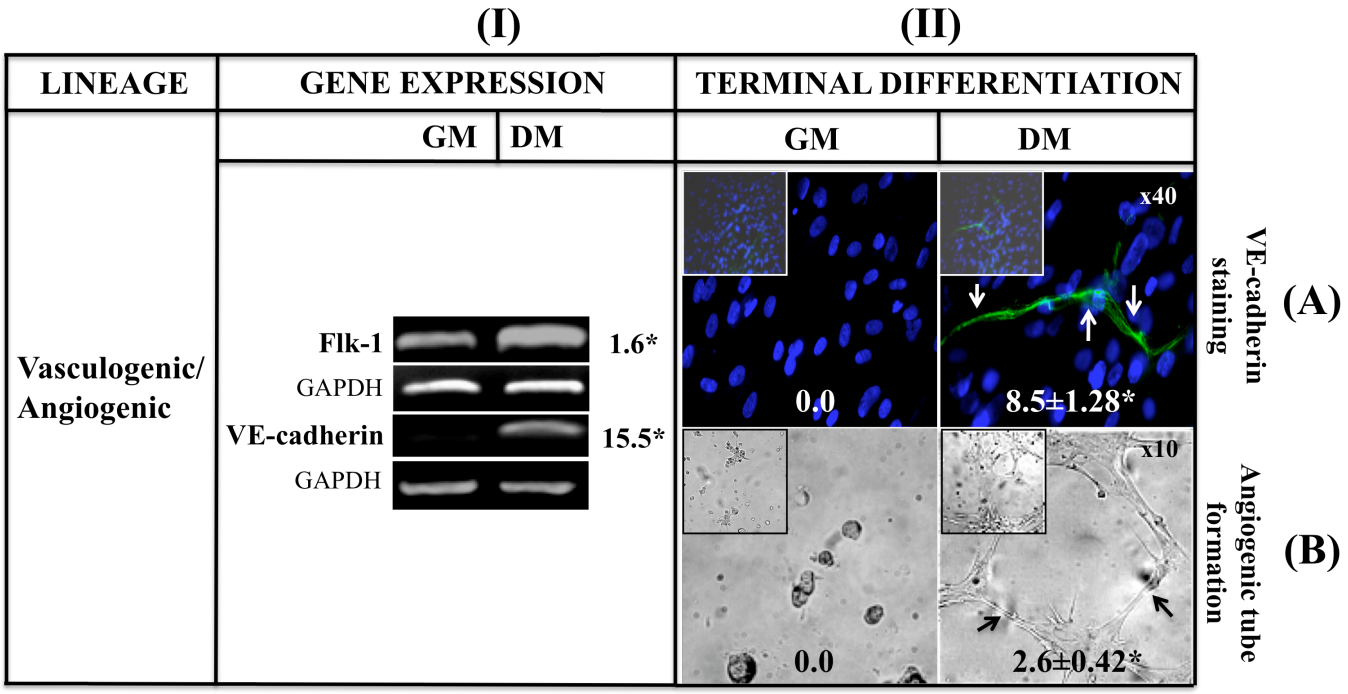


Figure 3.2. Vasculogenic/angiogenic differentiation of PDL cells in vitro.

(I) Representative RT-PCR gels of the expression of vasculogenesis/angiogenesis-associated genes by PDL cells cultured in GM and in vasculogenic/angiogenic DM. The values are the changes in PCR product band intensity obtained in the presence of DM compared with GM, defined as 1.0.

*(II) (A) Immunostaining of VE-cadherin after 5 weeks of culture in GM and in DM. The numbers are the % of VE-cadherin-positive cells. Nuclei are stained blue with Hoechst dye, and the white arrows show the green fluorescence-stained vasculogenic tube-like structures in the DM cultures. Magnification x40. (B) In vitro angiogenic structure formation by PDL cells cultured for 5 h in GM and in DM. The numbers are the angiogenic scores, and the black arrows show the closed polygonal structures formed in DM cultures. Magnification x10. The values are the means ±SE of triplicate measurements. *Indicates statistically significant difference compared with GM alone ($p<0.05$).*

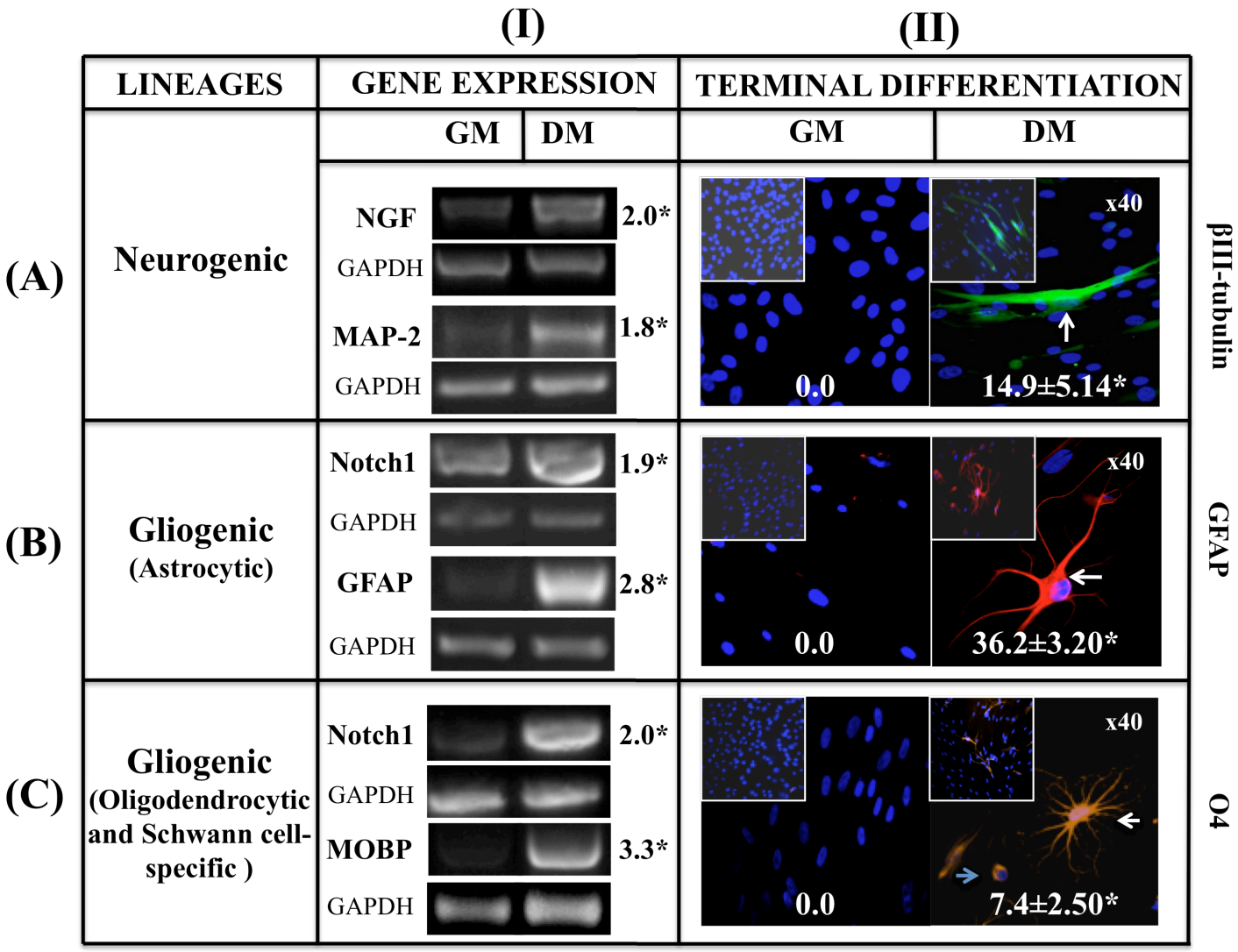


Figure 3.3. Neurogenic and gliogenic differentiation of PDL cells in vitro.

(I) Representative RT-PCR gels of the expression of neurogenesis and gliogenesis-associated genes by PDL cells cultured in GM and in neurogenic or gliogenic differentiation medium (DM). The values are changes in PCR product band intensity obtained in the presence of DM compared with GM, defined as 1.0.

(II) Immunostaining of (A) βIII-tubulin, (B) GFAP, and (C) oligodendrocyte-specific O4, in PDL cells cultured for 7 and 10 days in GM, in NM and GDM, respectively. The numbers are the % of βIII-tubulin, GFAP and O4-positive cells. Nuclei are stained blue with Hoechst dye, and the white arrow in (A) shows a green fluorescence-stained neuron-like cell, the white arrow in (B) shows a red fluorescence-stained astrocyte while, the white arrow in (C) shows the yellow fluorescence-stained oligodendrocyte-like cell blue arrow shows yellow fluorescence-stained Schwann-like cell. Magnification 40x. Nuclei are stained blue with Hoechst dye.

3.4. Discussion

A number of previous studies have suggested that the PDL may contain mesenchymal stem cell-like population capable of expressing a range of phenotypic markers such as STRO-1, CD29, CD44, CD105, CD106 and CD146 (Singhatanadgit et al., 2009; Tomokiyo et al., 2008; Xu et al., 2009; Cheng et al., 2009). However, these surface antigens are not definitive as they are also expressed by several other types of adult human cells, in addition to PDL cells, such as bone marrow-derived mesenchymal stromal cells, keratinocytes, T-lymphocytes and fibroblasts (Singhatanadgit et al., 2009; Mareddy et al., 2007; Staquet et al., 1989; Weninger et al., 2000; Pickl et al., 1997). Thus, the identification of PDL stem cells with multiple tissue regenerative capabilities has been limited partly due to the lack of definitive phenotypic markers of such cells. In addition, even though the complex process of homeostasis, repair and regeneration of PDL not only involves formation of mesenchymal tissues such as new bone and connective tissue architecture, but also the production of new BV, neurovascular channels and proprioceptive sensory nerve innervations, pivotal non-mesenchymal tissues present in the PDL (Maeda et al., 1999), the examination of the differentiation potential of these putative cell population has been limited to only mesenchymal (osteogenic, adipogenic and chondrogenic) lineages in previous studies (Singhatanadgit et al., 2009; Tomokiyo et al., 2008; Xu et al., 2009; Cheng et al., 2009). Thus, the present study investigated for the first time the multi-lineage (osteogenic, adipogenic, chondrogenic, myogenic, vasculogenic, angiogenic, neurogenic and gliogenic) differentiation potential of the heterogeneous primary PDL cell population *in vitro*.

Our findings showed that at least some cells obtained from PDL tissue, even after culture under non-selective growth conditions, constitutively expressed basal levels of all of the genes examined, except the vasculogenic/angiogenic VE-cadherin gene, indicating that 'mature' endothelial-like cells were unlikely to have been present in the population. In addition, under differentiation-inducing conditions we also showed that the adult PDL cells are capable of terminal osteogenic, adipogenic and chondrogenic differentiation *in vitro.* Similar observations were also reported using the adult human PDL clonal populations, which showed that the colony-forming cells within PDL have the ability to undergo osteogenesis, adipogenesis and chondrogenesis *in vitro* (Singhatanadgit et al., 2009). Moreover, CD146 and STRO-1-positive cells isolated from the heterogeneous PDL cell population were shown to be able to differentiate into the above mesenchymal lineages (Xu et al., 2009; Cheng et al.,

2009). In contrast, although under muscle-inducing conditions, PDL cells expressed a higher level of the late marker gene desmin and exhibited desmin-positive tube-like structures, these cells failed to express elevated levels of the pivotal early myogenic transcription factor MyoD1 and also the desmin-positive tube-like structure were found to be mononucleated. The reason for the lack of up-regulation of MyoD1 and the absence of multi-nucleated myotube-like structures is not yet clear, although similar observations have also been reported using BC_3H1 cells, a skeletal muscle cell line characterized by the lack of MyoD1 gene up-regulation, inability to fuse with surrounding cells and a non-multinucleated myotube phenotype (Brennan et al., 1990). The present results thus demonstrate that the PDL compartment contains progenitor/stem cell population capable of osteogenic, adipogenic and chondrogenic differentiation, although the presence of precursors capable of myogenic differentiation is less clear.

As with many other tissues (Nomi et al., 2002; Lammert et al., 2001; Matsumoto et al., 2001), vasculogenic and angiogenic differentiation processes are pivotal and indispensable features of tissue healing and regeneration, but have not previously been described for PDL cells. The results here have shown that transcripts of the Flk-1 and VE-cadherin genes, previously shown characteristics of endothelial cells (Heydarkhan-Hagvall et al., 2008), were both up-regulated when PDL cells were cultured in EBM-2 and, furthermore, that the PDL cells formed VE-cadherin-positive blood vessel-like structures, characteristics of vasculogenesis *in vitro*, similar to those previously reported observation using human adipose-derived stem cells and human umbilical cord-derived endothelial cells (HUVEC) (Heydarkhan-Hagvall et al., 2008; Ning et al., 2009). Moreover, although endothelial-like cells, a prerequisite for the process of angiogenesis, were not detected in the PDL cell population (based on the absence of VE-cadherin-positive cells in PDL cultures under non-differentiation-inducing conditions as noted above and also by the lack of expression of the endothelial cell surface marker CD34 shown previously (Singhatanadgit et al., 2009; Tomokiyo et al., 2008; Xu et al., 2009; Cheng et al., 2009)), when cultured on Matrigel the PDL cells were able to form angiogenic capillary-like tubules with minimal sprouting. Similar findings have also been reported using mesenchymal cell populations derived from human adipose tissue, murine embryo and human umbilical cord (Ning et al., 2009; Wang et al., 2010; Gang et al., 2006). The precise cellular origin of such capillary-like structures is not yet certain, since other reports have suggested that a fibroblastic cell population rather than the endothelial cells precursors might also be involved in the morphological appearance of

organized tubule network (Auerbach et al., 2003; Obeso et al., 1990). Thus, while the present findings provide evidence that the PDL contains precursor cells capable of undergoing vasculogenesis, it is possible that angiogenic structure formation *in vitro* by the heterogeneous PDL cell population could in part be due to the presence of fibroblastic-type cells.

PDL tissue has also been reported to contain dense proprioceptive sensory nerve innervations by the peripheral nervous system (Byers et al., 2004; Maeda et al., 1999; Johansson et al., 1991). Moreover, unlike the CNS in which oligodendrocytes and astrocytes are responsible for nerve myelination, sensory nerve innervations found in PDL tissue are myelinated by specialized glial (Schwann) cells as well as astrocytes (Byers et al., 2004; Maeda et al., 1999; Johansson et al., 1991). The present findings that, under neural-inductive conditions *in vitro* the neuronal marker genes NGF and MAP-2 were both up-regulated and cells were detected which exhibited a morphological appearance similar to bipolar peripheral sensory neurons containing an axon and one dendritic projection on opposite ends of the cell body (soma) (Kriegstein et al., 1983), indicates that the PDL cells might contain neuronal precursor cells. Similar findings were also reported using rat and human PDL cells in a neurosphere culture system (Techawattanawisal et al., 2007; Widera et al., 2007). In addition, PDL cells cultured under glial differentiation conditions were found here to have elevated levels of the astrocytic marker genes Notch1 and GFAP and the oligodendritic and Schwann cell-associated marker gene MOBP, to exhibit a GFAP-positive astrocyte-like cell morphology and, when supplemented with heregulinβ2, to exhibit O4 positive-oligodendrocyte and Schwann cell-like morphology (Maeda et al., 1999; Techawattanawisal et al., 2007; Widera et al., 2007). Astrocytic differentiation of these cells has previously been reported (Techawattanawisal et al., 2007; Widera et al., 2007), while the ability of PDL cells to form oligodendrocytes and Schwann cells has been shown here for the first time. It is notable that despite the histological absence of oligodendrocytes within PDL tissue (Byers et al., 2004), PDL cells *in vitro* nevertheless formed oligodendrocyte-like cells under appropriate differentiation conditions, suggesting that the PDL may contain primitive cells capable of undergoing all three types of glial cell differentiation *in vitro*. These results thus demonstrate that the PDL cells contain neuronal and glial precursor/stem cells capable of forming bipolar nerves and astrocytes, oligodendrocytes and Schwann cells under the appropriate differentiation conditions *in vitro*.

The summary of the results in Figure 3.4 shows that the adult human primary PDL cell population is capable of osteogenic, adipogenic and chondrogenic differentiation under the appropriate mesenchymal differentiation conditions *in vitro*. Such cells may possibly have a crucial role in PDL and AB regeneration during periodontal therapy. Moreover, PDL cells were also capable of undergoing vasculogenic differentiation indicating the presence of a primitive cell population capable of producing new blood vessels during the time of healing/repair/regeneration. The PDL compartment was also found to contain cells capable of producing bipolar nerve-like cells, one of the key elements of PDL proprioceptive function, and notably, these cells were also capable of producing all three types of glial cells which myelinate nerve axons.

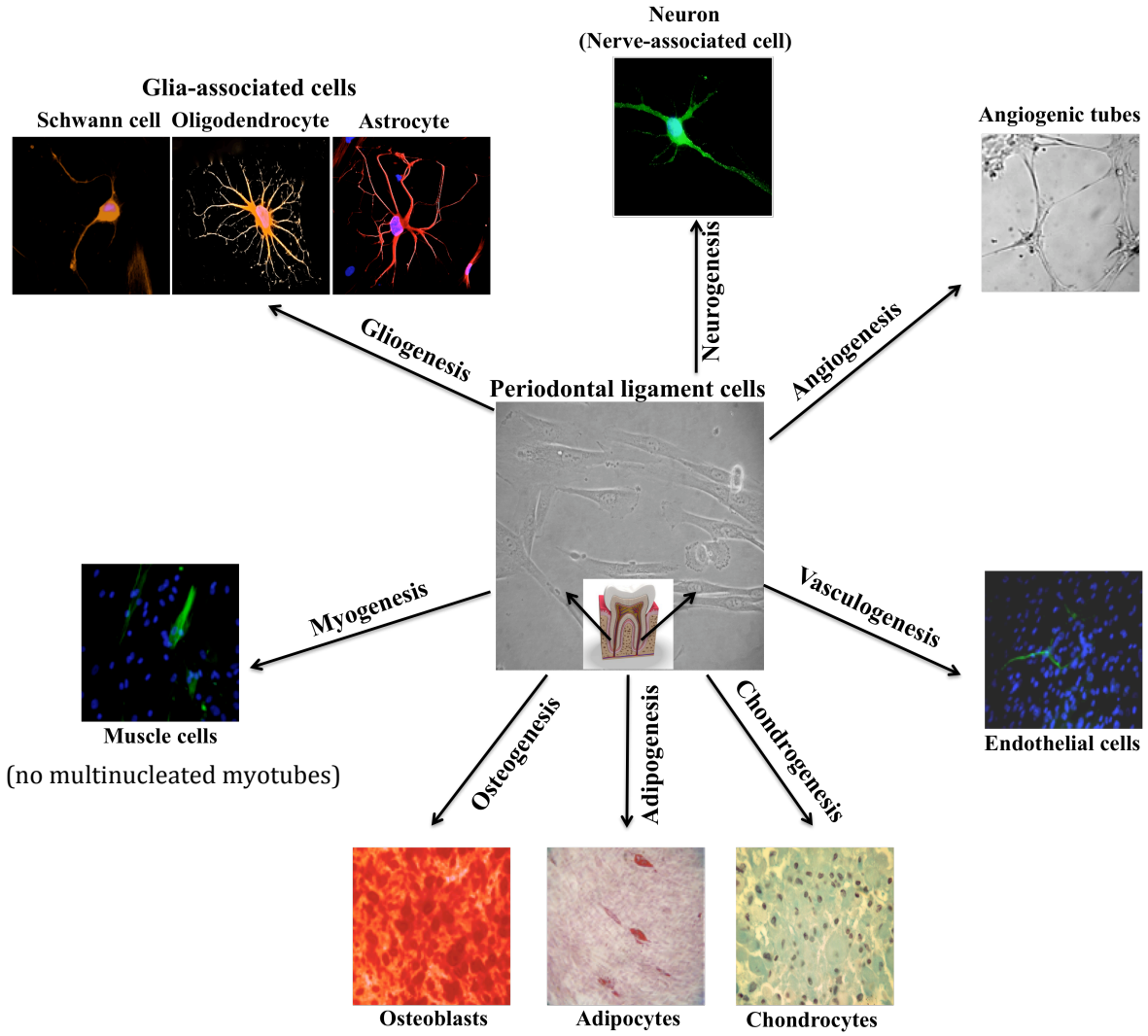


Fig. 3.4. Summary of multi-lineage differentiation potential of PDL cells. The representative micrograph in the centre shows adult human PDL cells, obtained originally from the PDL of the tooth (black arrows), during culture in non-selective conventional GM.

Chapter 4

4.1. Introduction

Amelogenins are a family of proline-rich peptides formed from the amelogenin gene by alternative splicing and post-translational modifications (Fincham et al, 1993), and include the 5 kDa tyrosine-rich (TRAP) and 6-8 kDa leucine-rich (LRAP) isoforms (Fincham et al, 1993). They are the constituents of crude preparation EMP and commercially available heat-treated EMD and have been extensively studied partly because of their effect on osteogenic differentiation *in vitro* and bone regeneration *in vivo* (Lossdorfer et al, 2007; Sculean et al, 2008; Reseland et al, 2006; Keila et al, 2004; Sculean et al, 2004; Donos et al., 2003). Thus, EMP and EMD have been reported to promote osteogenesis by up-regulating bone-associated genes such as alkaline phosphatase (ALP), osteopontin (OP), osteocalcin (OC) and bone sialoprotein (BSP), and by stimulating the production of bone-like mineralized nodules *in vitro* (Lossdorfer et al, 2007; Keila et al, 2004). However, other studies have shown that these preparations down-regulated these bone-associated markers and terminal osteogenic differentiation of human periodontal ligament (PDL) cells, osteoblasts and bone marrow derived mesenchymal stem cells (BMSC) (Wada et al, 2008; Hama et al, 2008). Similarly, while some experimental models and clinical studies have demonstrated that, *in vivo*, EMP and EMD promote regeneration of periodontal ligament (PDL) and root cementum, its effect on new bone growth has often been inconsistent and unpredictable (Donos et al, 2005; Donos et al., 2003; Heijl et al, 1997). Although the reason for these discrepancies is not yet clear, it may be due at least partly to the use of different preparations of EMP and EMD, some of which have been reported to contain BMP- and TGFβ-like activities (Johnson et al, 2009; Suzuki et al, 2005). In addition, such preparations are highly heterogeneous and contain several different proteins and their isoforms, as noted above, which are likely to differ both qualitatively and quantitatively between different preparations.

Recent analysis of a commercially available heat-treated form of EMP (Emdogain®; EMD, Institut Straumann, Basel, Switzerland) has demonstrated the presence of two main sub-fractions (Mumulidu et al, 2007). One comprises mainly the amelogenin-derived TRAP peptide and the other contains > 6 kDa peptides, including the amelogenin peptide LRAP, sheathlin and full-length amelogenin (Fincham et al, 1993). While sheathlin has been shown to have a role in cementum development and repair (Kanazashi et al., 2006), the effects of these amelogenin peptide-containing sub-fractions of EMD on osteogenesis have not been

investigated. The present study has therefore examined the response of bone-forming cells to EMD components under conditions that facilitate terminal osteogenic differentiation *in vitro*.

4.2. Materials and methods

PDL cell culture and RT-PCR were performed as described in Chapter 2; additional methods used in this chapter are described below.

4.2.1. Isolation of primary human alveolar bone (AB) cells

Primary human bone cells (AB) were grown from cortico-lamellar bone of the maxilla of patients undergoing routine extractions at the Eastman Dental Hospital, as previously described (Singhatanadgit et al, 2006). Briefly, the bone chips were cut into approximately 1-2 mm^3 pieces, placed in 48-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 15 min to enable them to adhere to the culture plates. Low glucose-Dulbecco's Modified Eagle's Medium (Low glucose-DMEM) (Gibco) containing 10% FCS (PAA Laboratories) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, and 2 mM L-glutamine (all from Gibco) was then added and the explants were cultured until the outgrowth of the adherent cells reached confluence. Cells were used between passage 2 and 5 in the experiments.

In addition to the PDL and AB cells, bone marrow-derived mesenchymal stem cells (BMSC) (Lonza, Slough, UK) and a recently isolated 'clonogenic' cell population from adult human PDL which was shown to exhibit characteristic features of a bone progenitor/stem cell (clone 7; Singhatanadgit et al, 2009), were also used in the present study in order to examine the effects of EMD components on a range of bone-forming cells. BMSC and clone 7 were cultured in the same way as the PDL and AB cells, described above, and used between passage 3 and 6.

4.2.2. Treatment of cells with EMD and the EMD Fractions

EMD, the EMD-derived sub-fraction < 6 kDa (Fraction C) and the EMD-derived > 6 kDa sub-fraction (Fraction A) were diluted in 0.1% acetic acid and added directly to the cells when they reached approximately 90% confluence. EMD and Fractions C and A were used at a final concentration of 100 μg/ml throughout the experiments, unless otherwise noted. These concentrations were determined from their dose effects on terminal PDL cell osteogenesis *in vitro,* as described below.

4.2.3. Effects of the EMD, Fraction C and Fraction A on terminal differentiation of bone-forming cells

PDL cells were seeded into 24-well plates at a density of 2.5 x 10^4 cells/well and cultured in non-selective growth medium (GM) containing low glucose-DMEM supplemented with 10% FCS, 200 U/ml penicillin and 200 μg/ml streptomycin) for 3 days. Osteogenic medium (OM) was then added, consisting of GM supplemented with 0.1 mM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate and 10 nM dexamethasone (all from Sigma). 1, 3, 10, 30 and 100 μg/ml concentrations of EMD, Fraction C and Fraction A were added to the cultures and the medium was changed every 3 days.

Similarly, AB, BMSC and clone 7 were also seeded into 24-well plates at a density of 2.5 x 10^4 cells/well and cultured in GM for 3 days until 90% confluent, the medium then replaced with OM containing EMD, Fraction C and Fraction A (all 100 μg/ml) and was changed every 3 days. Control cultures were incubated with OM alone.

The formation of mineralized nodules, a measure of terminal osteogenic differentiation, was determined by alizarin red S staining of calcium-containing deposits, as follows. After 3 weeks of culture in OM in the presence of EMD, Fraction C and Fraction A cells were fixed with 10% formaldehyde for 15 min and washed with distilled water. The samples were then incubated with 2% Alizarin Red S (pH 4.2) (Sigma) for 15 min at room temperature, washed with ice-cold methanol, air-dried and photographed. The level of alizarin red staining was quantified by absorbance at 562 nm (A_{562}), after eluting the stain for 2 h with 10% cetylpyridinium chloride in 10 nM sodium phosphate buffer, pH 7.0.

4.2.4. Quantitative-polymerase chain reaction (Q-PCR) analysis of osteogenesis-associated genes

PDL cells were seeded into 24-well plates at a density of 2.5 x 10^4 cells/well and cultured in GM for 3 days. GM was then replaced with OM and the cells cultured in the presence of EMD, Fraction C and Fraction A, and the medium changed every 3 days. On days 4, 7, 10 and 14, total RNA was extracted and Q-PCR carried out to measure the 'early' osteogenic marker gene runt-related transcription factor-2 (Runx2) (day 4), the 'intermediate' osteogenic marker gene osteopontin (OP) (day 7) and the 'late' osteocalcin (OC) (day 14) and bone sialoprotein (BSP) (day 14) genes, as described below.

Total RNA was isolated from PDL cells using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturers instructions. For reverse transcription first strand cDNA was synthesized using 1 μg of total RNA, as previously described (Singhatanadgit et al, 2009), with primers obtained from Applied Biosystems (Applied Biosystems, Foster City, CA). Q-PCR analysis was carried out using the ABI Prism® 7300 sequence detector (Applied Biosystems), the Taqman® Gene Expression Assay consisting of the unlabelled specific PCR primers and Taqman® MGB probes with FAM™ dye labeling in a 96-well plate format. Thermal cycler conditions were used as recommended by the manufacturer and the data were collected and analyzed by the SDS 1.2 software (Applied Biosystems). All PCR reactions were performed in triplicate and each of the gene cycle threshold (ct) value normalized to the GAPDH ct values detected simultaneously on the same plate.

4.2.5. ALP activity assay

PDL cells were cultured in OM with and without EMD, Fraction C and Fraction A, with the medium changed every 3 days. Since ALP is involved in a late phase of mineralization (Singhatanadgit et al., 2006) it's activity in PDL cells was measured on day 14, as described previously (Singhatanadgit et al, 2006). Briefly, triplicate wells were washed with PBS and the cells incubated with 200 μl of 5 mM *p*-nitrophenyl phosphate in 50 mM glycine, 1 mM $MgCl_2$ and 150 mM 2-amino-2-methyl-1-propanol buffer, pH 10.5, at 37^{o}C for 1 h. A 50 μl aliquot of 3 M NaOH was added to stop the enzymatic reaction and the absorbance measured at 405 nm (A_{405}).

4.2.6. Effects of a BMP-antagonist noggin and a TGFβ-antagonist UB-505124 on EMD, Fraction C and Fraction A-mediated PDL osteogenesis

To determine whether EMD, Fraction C and Fraction A act via a BMP and/or TGFβ-dependent pathway, the effects of noggin, a BMP pathway inhibitor (Chen et al., 2004; Miyazono, 2000), and UB-505124, a TGFβ pathway inhibitor (DaCosta et al., 2004; Miyazono, 2000), on PDL osteogenesis was measured, as described below. PDL cells were plated and cultured in OM for 2 weeks in the presence of EMD, Fraction C and Fraction A. Replicate test cultures also contained 100 ng/ml of noggin (Peprotech, London, UK) or 1 μM

UB505124. After 2 weeks, the cultures were stained with alizarin red S and the formation of mineralized nodules measured as described above.

4.2.7. Effects of EMD, Fraction C and Fraction A on the Smad pathway of osteogenic regulation

Smad1/5/8 is an inactive cytoplasmic protein complex that is activated by phosphorylation to p-Smad1/5/8 and then translocated to the nucleus where it acts as a transcription factor for the BMP gene, thereby stimulating osteogenic activity (Rider et al., 2010; Miyazono, 2000). To determine the effects of EMD, Fractions C and Fraction A on Smad1/5/8 activation to p-Smad1/5/8, flow cytometry (FCM) was used to examine changes in the relative levels of p-Smad1/5/8 and immunocytochemistry was carried out to examine changes in intracellular localization, as described previously (Amin et al., 2011; Singhatanadgit et al, 2006).

Briefly, PDL cells were incubated in 24-well plates in GM for 3 days, GM was then replaced with low glucose-DMEM with 1% FBS for a further period of 48 h in order to reduce the levels of endogenous growth factors. In order to eliminate the presence of any exogenous growth factors, the culture medium was replaced with (1) completely serum-free OM containing low glucose-DMEM, 0.1 mM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate and 10 nM dexamethasone (all from Sigma), (2) serum-free OM + EMD (100 μg/ml), (3) serum-free OM + Fraction C (100 μg/ml) and (4) serum-free OM + Fraction A (100 μg/ml), and further cultured for 1 h. For the FCM, after treatment of the PDL cells with EMD, Fraction C and Fraction A as above, the cells were detached with 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA (trypsin-EDTA) for 5 min at RT, fixed with 1% paraformaldehyde, permeabilised with 0.1% Triton X and blocked for 20 min using 10% NGS. Cells were then incubated with 1:100 rabbit polyclonal anti-p-Smad1/5/8 (Insight Biotechnology, London, UK) for 1 h. After washing, the cells were further incubated with FITC-conjugated goat anti-rabbit (1:200) for 1 h at RT. FITC- conjugated normal rabbit IgG was used as a negative control. The cells were centrifuged and re-suspended in 200 μl of PBS and the fluorescence intensity of 10,000 individual cells measured using a flow cytometer (FACScan; Becton-Dickinson, Cowley, UK). Data analysis was carried out using the WinMDI 2.8 software program.

For immunocytochemistry, after treatment of the PDL cell monolayers with EMD, Fraction C and Fraction A as above, the cells were fixed with 4% paraformaldehyde for 15 min at RT and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with 10% NGS in PBS for 1 h, followed by incubation for 1 h at RT with 1:100 rabbit polyclonal anti-p-Smad1/5/8 (Insight Biotechnology). After washing, FITC-conjugated goat anti-rabbit was added (1:200) for 1 h at RT. Nuclei were stained with Hoechst stain, and p-Smad1/5/8-positive cells were visualized by their green fluorescent staining.

Smad6 is a negative regulator of BMP-mediated osteogenic differentiation, exerting its activity by binding to and inhibiting the phosphorylation of the Smad1/5/8 complex (Wu et al., 2004). This prevents translocation to the nucleus and thereby blocks its function as a BMP transcription factor, as noted above (Chen et al., 2004). To examine the effects of EMD, Fraction C and Fraction A on the Smad6 gene, the PDL cells were cultured in OM in the presence of these proteins (all used at 100 μg/ml) and gene expression assessed by conventional PCR on day 4, as previously described (Amin et al, 2011). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene was used as an endogenous control. The sequences of the forward and reverse primers (from Sigma-Genosys, Pampisford, UK): Smad6 forward: AAAGACGCACTTTGGCTTA, reverse: CGAATACTTTATTATCGAGTGACTG; GAPDH forward: CCACCCATGGCAAATTCCCATGGCA, reverse: CTGGACGGCAGGTCAGGTCCACC. Smad6 primers were used at an annealing temperature of 57^{o}C for 30 cycles and GAPDH primers were used at an annealing temperature of 55^{o}C for 25 cycles.

4.2.8. Identification of the bioactive components of EMD, Fraction C and Fraction A

As noted above, TRAP is a naturally-occurring 45-amino acid proteolytic product of the full-length amelogenin protein, and is the major component of Fraction C (Mumulidu et al., 2007; Fincham et al., 1993). LRAP is a naturally-occurring 56-amino acid spliced form of amelogenin, and is a major component of Fraction A, as previously reported (Mumulidu et al., 2007; Fincham et al., 1993). We have identified that TCT is the unique 12-amino acid C-terminal sequence present in TRAP, while LCT is the unique 23-amino acid C-terminal sequence present in LRAP. TCT and the parent peptide TRAP, and LCT and the parent peptide LRAP were chemically synthesized (Insight Biotechnology) (Table 4), diluted in cell culture grade water (Thermo Scientific, Basingstoke, UK) and 1 μg/ml of each added directly

to the PDL cells, clone 7, AB cells and BMSC when they reached approximately 90% confluence. These concentrations were determined from their dose effects (from 0.1-100 μg/ml) on terminal osteogenesis on PDL cells *in vitro*, as described above.

4.2.9. Statistical analysis

The PCR data are shown as the mean fold-change ± standard error (± SE) of three separate experiments compared with that of control cells cultured in OM alone (defined as 1.0). Alizarin red staining intensity is shown as the relative staining intensity ± SE of three separate experiments compared with OM alone (defined as 1.0). FCM data is presented as the mean average fluorescence intensity (AFI ± SE) of three separate experiments. One-way ANOVA was used to assess statistical significant difference followed by the post-hoc Bonferroni test for multiple comparisons between the means ($p<0.05$) (SPSS 11.0 software, Chicago, IL).

4.3. Results

4.3.1. Effects of EMD and the EMD Fractions on terminal differentiation of bone-forming cells

Increasing concentrations of the EMD preparation used in the present study were found to progressively stimulate the formation of bone-like mineralized nodules by PDL cells, a key indicator of their ability to undergo terminal osteogenic differentiation *in vitro* (Fig. 4.1), with 100 μg/ml of EMD resulting in a 430% increase in alizarin red staining intensity compared with control PDL cells cultured in OM alone ($p<0.05$). In marked contrast, increasing concentrations of Fraction C decreased alizarin red staining, with 100 μg/ml resulting in a 30% reduction in staining intensity ($p<0.05$; Fig. 4.1). Notably, as with EMD, increasing concentrations of Fraction A progressively increased terminal osteogenic differentiation of the PDL cells, with 100 μg/ml resulting in a level that was 680% higher than found when the cells were cultured in OM alone, as shown in Figure 4.1 ($p<0.05$). These results thus show that 100 μg/ml of EMD and Fraction A are stimulatory, whereas 100 μg/ml of Fraction C is inhibitory to terminal osteogenic differentiation of PDL cells. Since EMD and the EMD Fractions were found to have the most pronounced effect on PDL osteogenesis at 100 μg/ml, this concentration was used throughout the experiments reported below.

EMD and the EMD Fractions were also found to have similar effects on other bone-forming cells, as shown in Fig. 4.1. Thus, when PDL clone 7 (an osteogenic precursor/stem cell-like clone isolated from adult human PDL; Singhatanadgit et al., 2009) was cultured in OM in the presence of 100 μg/ml of EMD, there was a significant increase (220%; $p<0.05$) in alizarin red staining intensity observed compared with OM alone. In contrast, in the presence of Fraction C the staining intensity decreased by 40% compared with OM alone ($p<0.05$) while, as with EMD, the presence of Fraction A resulted in a 450% increase in staining intensity (Fig. 4.1). Similar effects on mineralization were also observed with both AB and BMSC cells, which were stimulated by EMD (staining intensity 2.6 and 2.0, respectively; $p<0.05$) and also by Fraction A (370% and 280% increase, respectively; $p<0.05$) compared with OM alone. In contrast, Fraction C reduced the staining intensities of these two types of bone-forming cell by 30% and 40%, respectively, compared with OM alone ($p<0.05$). Thus the differential effects of the EMD Fractions were exerted not only on the heterogeneous PDL cell population but also on the isolated osteogenic clone, AB cells and the BMSC.

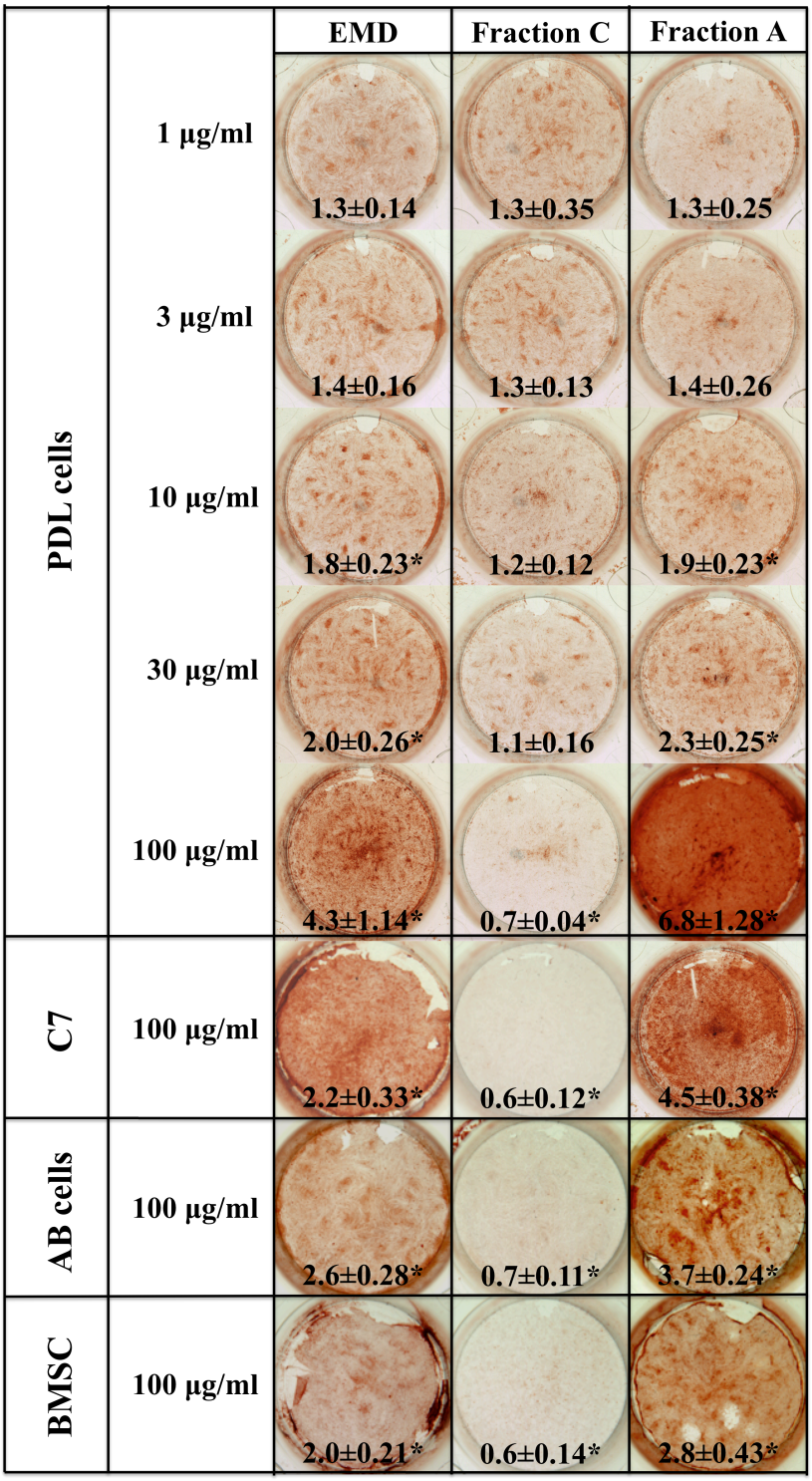


Figure 4.1. Effects of EMD, Fraction C and Fraction A on terminal osteogenic differentiation of PDL and other bone-forming cells. Alizarin red staining of the cells cultured for 3 weeks in OM in the presence of EMD, Fraction C and Fraction A at concentrations 1, 3, 10, 30 and 100 µg/ml for the PDL cells and 100 µg/ml for the PDL clone 7, AB and BMSC. The numbers are the relative staining intensities compared with OM alone, defined as 1.0.

**Indicates statistically significant difference compared with OM alone $p<0.05$.*

4.3.2. Effects of EMD, Fraction C and Fraction A on the expression of bone-associated markers

The results in Table 3 show that EMD significantly up-regulated the expression of the 'early' osteogenic transcription factor Runx2 after 4 days (approximately 2-fold), whereas the presence of Fraction C had no effect compared with OM alone. As with EMD, Fraction A also up-regulated Runx2 nearly 2-fold ($p<0.05$) (Table 3). Similarly, the intermediate OP and late osteogenic marker genes OC and BSP, and ALP activity were all found to be significantly up-regulated by both EMD and Fraction A, whereas Fraction C significantly down-regulated all of these genes except for OP (Table 3). These results thus demonstrate that although Fraction C had no effect on the early Runx2 and intermediate OP genes, it nevertheless suppressed the key late markers OC, BSP and ALP, and it also suppressed the terminal osteogenic differentiation of PDL and other bone-forming cells as described above. Moreover, it also suggests that Fractions C and A, two different components of EMD, have completely opposite effects on bone-forming cells.

Table 3. Effects of EMD and EMD Fractions on osteogenic marker expression

Osteogenic markers		Relative expression[§]		
		EMD	Fraction C	Fraction A
Early	**Runx2 gene** (4 days)	1.97±0.23*	0.95±0.16	1.93±0.20*
Inter-mediate	**OP gene** (7days)	3.78±0.18*	1.03±0.10	5.13±0.30*
Late	**OC gene** (10 days)	2.34±0.10*	0.86±0.10*	3.11±0.24*
	BSP gene (14 days)	5.42±0.75*	0.38±0.32*	22.86±2.32*
	ALP enzyme activity (14 days)	1.46±0.15*	0.72±0.12*	1.94±0.22*

PDL cells were cultured in OM in the presence of EMD, Fraction C and Fraction A for up to 2 weeks. Q-PCR analysis was carried out for the early osteogenic transcription factor Runx2, intermediate matrix marker gene OP and the late matrix marker genes OC and BSP. ALP activity was also measured at day 14.

[§]The values shown are relative to the values obtained with OM alone, defined as 1.0

*Indicates statistically significant difference compared with OM alone ($p<0.05$)

4.3.3. Effects of noggin and UB505124 on EMD, Fraction C and Fraction A-mediated osteogenesis

To determine whether EMD and the EMD Fractions act by modulating the BMP- and TGFβ-mediated pathways of osteogenic differentiation, the PDL cells were incubated in OM in the absence and presence of noggin, a BMP pathway inhibitor, and UB505124, a TGFβ pathway inhibitor. The results in Figure 4.2 show that the presence of noggin or of UB505124 alone had no effect on the alizarin red staining intensity of control PDL cells cultured in OM. As before, when the cells were cultured in the presence of EMD the staining intensity significantly increased to 2.1. However, the concurrent presence of noggin or UB505124 in the EMD cultures markedly reduced the alizarin red levels compared with cultures incubated with EMD alone (from 2.1 to 1.0 and 1.4, respectively; $p<0.05$) (Fig. 4.2). Moreover, the presence of noggin and UB505124 similarly further reduced the low mineralization activity obtained in the presence of Fraction C alone (0.1 and 0.3, respectively, compared with 0.6; $p<0.05$). Notably, the high level of mineralization observed in the presence of Fraction A (3.5) was unaffected by noggin and UB505124 (3.1 and 3.1, respectively). These results thus suggest that the osteogenic activity of EMD and Fraction C, but not of Fraction A, is mediated via BMP and TGFβ-dependent pathways.

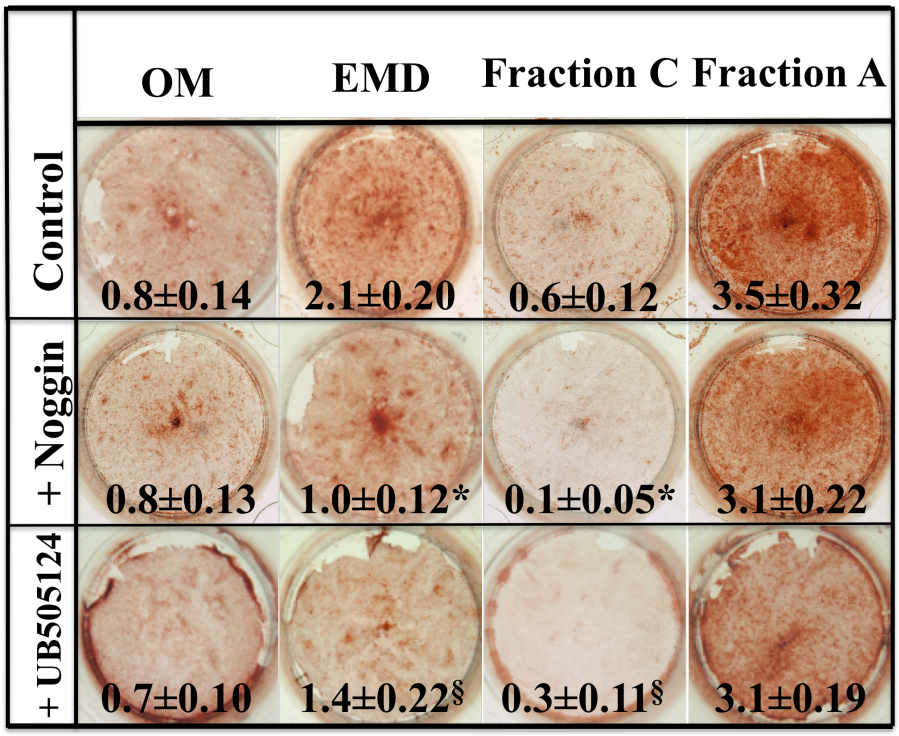


*Figure 4.2. Alizarin red staining of PDL cells cultured for 14 days in OM in the presence of EMD (100 µg/ml), Fraction C (100 µg/ml) and Fraction A (100 µg/ml) with and without noggin and UB505124. The numbers are the alizarin red staining intensity. *Indicates statistically significant difference between noggin-treated verses untreated ($p<0.05$); §Indicates statistically significant difference between UB505124-treated verses untreated ($p<0.05$).*

4.3.4. Effects of EMD, Fraction C and Fraction A on the Smad pathway of osteogenic regulation

The Smad pathway has previously been shown to modulate BMP-mediated osteogenic differentiation (Singhatanadgit et al., 2006; Miyazozo, 2000). In view of the apparent effect of EMD and the EMD Fractions on the BMP pathway, shown above, the effect of these proteins on the relative level of p-Smad1/5/8, the active form of the BMP transcription factor, was examined by FCM. As shown in Figure 4.3, EMD and Fraction A were found to have no effect on p-Smad1/5/8 compared with serum-free OM alone, whereas the presence of Fraction C significantly reduced this activated complex by 42% ($p<0.05$). Moreover, immunocytochemical studies demonstrated that approximately 50% of the cells in the presence of serum-free OM alone and with EMD and Fraction A expressed p-Smad1/5/8, whereas in the presence of Fraction C no p-Smad1/5/8-positive cells were detected (Fig. 4.4). These results thus indicate that Fraction C inhibits osteogenic differentiation via suppression of activated p-Smad1/5/8.

Smad6 has previously been shown to be a negative regulator of BMP-mediated osteogenesis that exerts its activity by binding to and preventing the phosphorylation and subsequent nuclear translocation of Smad1/5/8. To determine whether the apparent inhibitory effect of Fraction C on the level of p-Smad1/5/8, as shown above, was mediated via Smad6, the expression of the Smad 6 gene was examined in the absence and presence of EMD and the EMD Fractions. The results in Figure 4.5 show that while EMD and Fraction A did not cause any marked change in Smad6 gene expression, in the presence of Fraction C the Smad6 gene was significantly up-regulated (2-fold; $p<0.05$) compared with OM alone, These results thus suggest that Fraction C may inhibit osteogenic activity via stimulating Smad6, a negative regulator of the BMP pathway.

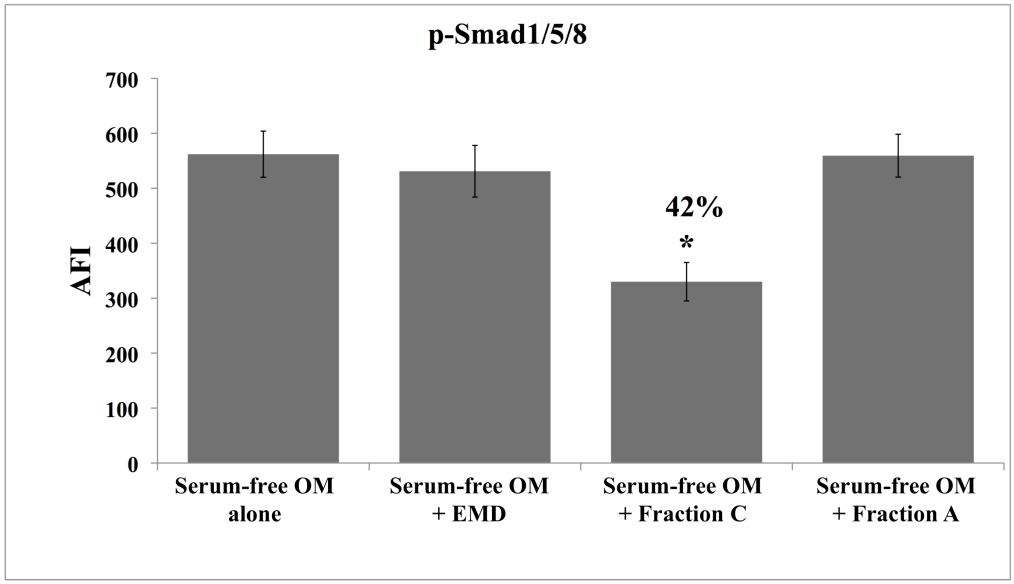


Figure 4.3. FCM analysis of the effects of EMD and the EMD Fractions on intracellular levels of p-Smad1/5/8. Cells were immunostained for p-Smad1/5/8 after 1 h of incubation in the presence of 100 µg/ml of EMD, Fraction C and Fraction A. The numbers show the AFI of 10,000 cells cultured in the presence of serum free OM alone and in the presence of EMD, Fraction C and Fraction A, as described in ***Materials and methods****. *Indicates a statistically significant decrease of 42% compared with OM alone ($p<0.05$)*

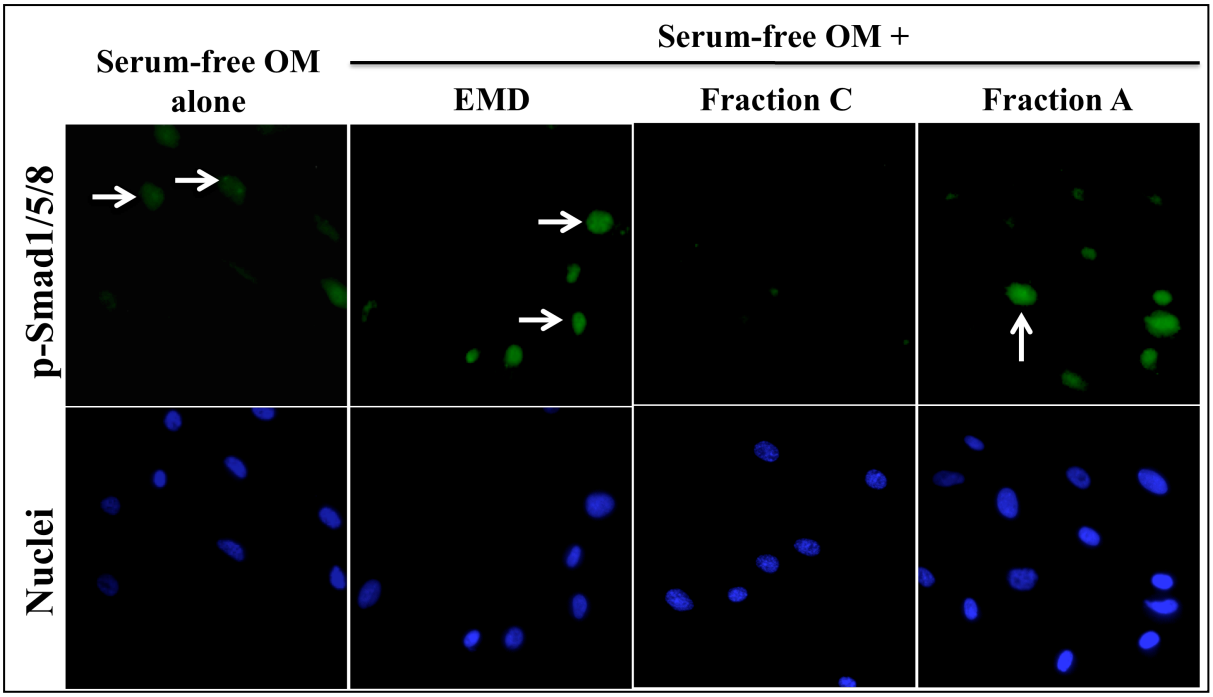
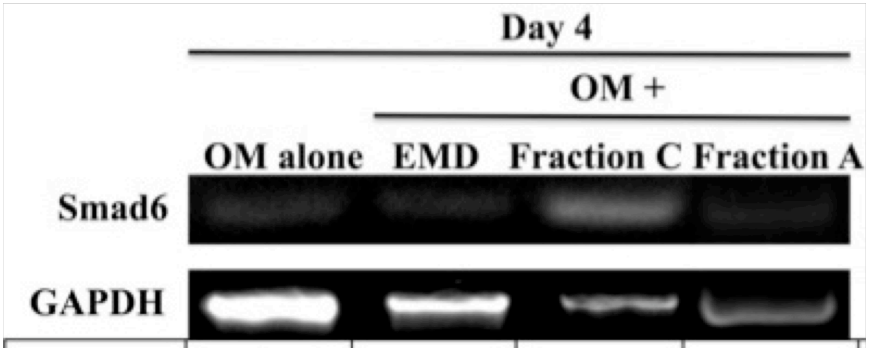


Figure 4.4. Effects of EMD and the EMD Fractions on p-Smad1/5/8 localization. Immunostaining of p-Smad1/5/8 after 60 min of incubation of PDL cells in serum free OM in the absence and presence of 100 µg/ml of EMD, Fraction C and Fraction A. Nuclei are stained blue with Hoechst dye, and the white arrows show the green fluorescence-stained PDL cells positive for p-Smad1/5/8. Note the nuclear localization of p-Smad1/5/8.



Band intensity relative to GAPDH	0.25	0.30	**0.50**	0.34
Relative gene expression	1.0±0.10	1.2±0.07	**2.0±0.14***	1.2±0.10

*Figure 4.5. Effects of EMD and the EMD Fractions on Smad6 gene expression. Representative RT-PCR gel showing the expression of Smad6 transcripts of PDL cells cultured in OM in the presence of 100 μg/ml of EMD, Fraction C and Fraction A. GAPDH was used as an endogenous control to normalize the gene expression. The numbers below the representative electrophoresis gels show the fold induction of the EMD and the EMD Fractions treated cells compared with control cells cultured in OM alone, defined as 1.0. *Indicates statistically significant difference compared with OM alone ($p<0.05$)*

4.3.5. Effects of synthetic amelogenin peptides on bone-forming cells

The TRAP and LRAP peptides are major components of Fractions C and A, respectively, as noted above. In view of the effects of these Fractions on osteogenic differentiation, chemically synthesized TRAP and LRAP were obtained and examined for their effect on the terminal osteogenic differentiation of bone-forming cells. As with Fraction C, synthetic TRAP (1 μg/ml) significantly reduced bone-like nodule formation and the alizarin red staining intensity of the PDL cells, clone 7, AB cells and BMSC (40-60% reduction; $p<0.05$) compared with OM alone (Figure 4.6). In contrast, as with Fraction A, 1 μg/ml of LRAP markedly stimulated bone-like nodule formation and increased the staining intensity of the PDL cells, clone 7, AB cells and BMSC (between 250 and 440% increases; $p<0.05$) compared with OM alone. These results thus demonstrate that the chemically synthesized amelogenin isoforms TRAP and LRAP exhibit activities that are similar to EMD Fractions C and A and further, these data suggest that TRAP and LRAP are the active components of EMD that differentially regulate osteogenic differentiation.

Alignment of the TRAP and LRAP isoforms shows that the 33-amino acid sequence at the N-terminal of TRAP and of LRAP are common to each other (Table 4). In view of the differential activities of TRAP and LRAP, it was considered that this overlapping sequence is likely to be devoid of the specific bone-modulating activity exhibited by TRAP and LRAP. In experiments not reported here, we found that concentrations between 0.1-100 μg/ml of this 'null' peptide had no effect on cell mineralization *in vitro* (Appendix Material 1.1). In marked contrast, however, the 12-amino acid C-terminal peptide (TCT) unique to the osteogenesis-inhibitory TRAP, and the 23-amino acid C-terminal peptide (LCT) unique to the osteogenesis-stimulatory LRAP, were found to have potent but opposite osteogenic effects, as shown in Figure 4.6. Thus, 1 μg/ml of TCT was found to strongly inhibit (between 30 and 50% reduction; $p<0.05$), whereas 1 μg/ml of LCT strongly stimulated, terminal osteogenic differentiation of PDL cells, clone 7, AB cells and BMSC (between 240 and 590% increases; $p<0.05$), compared with OM alone (Fig. 4.6). These results indicate that the unique C-terminal amino acid sequences of TRAP and LRAP, TCT and LCT respectively, are the bioactive sequences responsible for the differential activities of the parent amelogenin isoforms.

Table 4. Amelogenin-derived peptide sequences	
TRAP (45 amino acids)	MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHP YTSYGYEPMGGW
LRAP (56 amino acids)	MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHP SLLPDLPLEAWPATDKTKREEVD
TCT (12 amino acids)	YTSYGYEPMGGW
LCT (23 amino acids)	SLLPDLPLEAWPATDKTKREEVD

Amino acid sequences: $-NH_2 \longrightarrow -COOH$

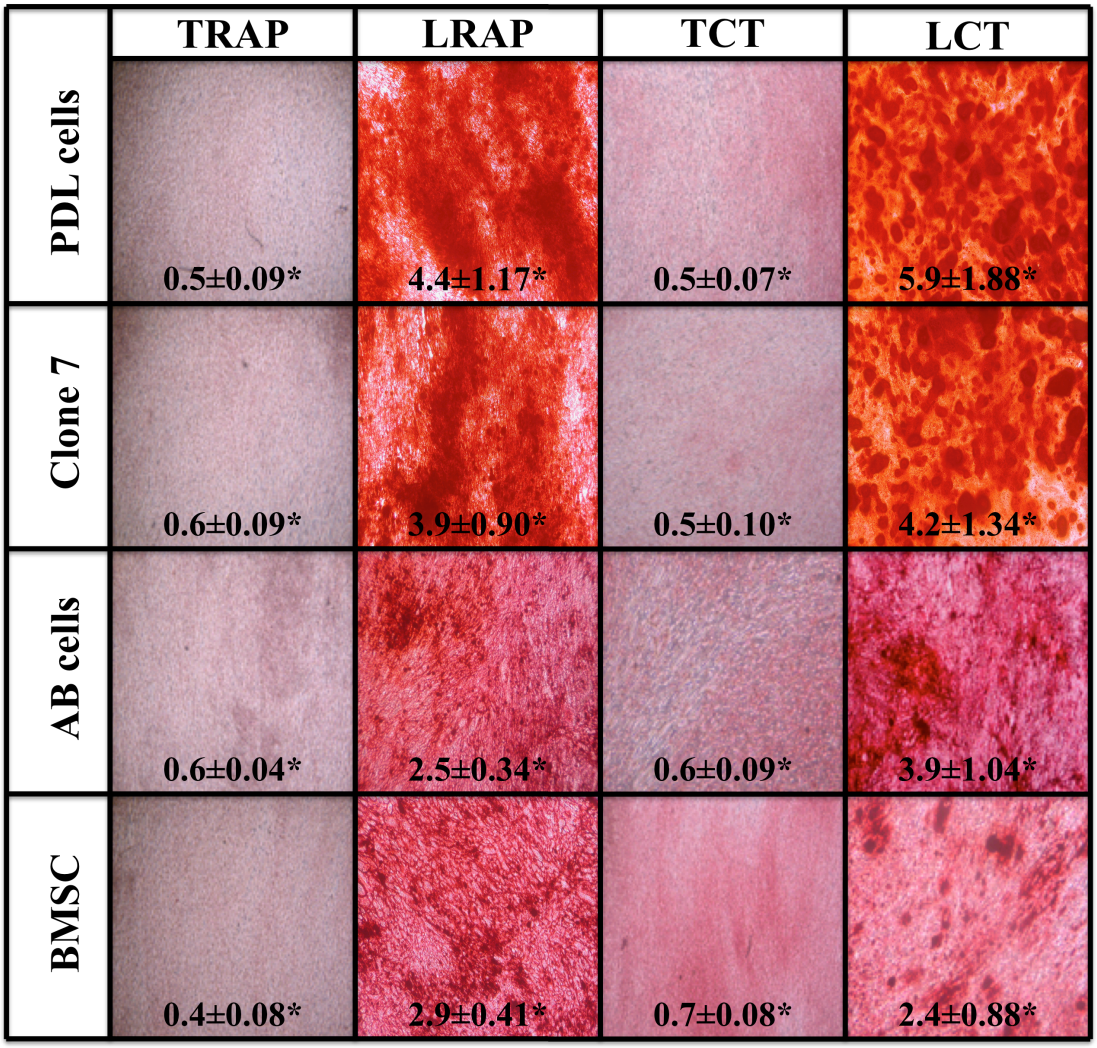


*Figure 4.6. Effects of synthetic amelogenin isoforms and peptides on bone-forming cells. Alizarin red staining intensity of the PDL cells, clone 7, AB cells and BMSC cultured for 3 weeks in OM in the presence of synthetic TRAP (1 μg/ml), LRAP (1 μg/ml), TCT (1 μg/ml) and LCT (1 μg/ml). The numbers are the relative staining intensity compared with OM alone, defined as 1.0. *Indicates statistically significant difference compared with OM alone ($p<0.05$)*

4.4. Discussion

A number of studies, including the present, have demonstrated that commercial preparation of EMD and crude preparation of EMP stimulate bone-associated markers and bone-like nodule formation of PDL and other bone-forming cells (Gestrelius et al., 1997; Van der pauw et al. 2000; Nagano et al. 2004). In contrast, EMD and EMP have also been reported to inhibit the expression of osteogenic genes and mineralized bone-like nodule formation *in vitro* (Hamma et al., 2008; Hakki et al., 2001) and also *in vivo* (Donos et al, 2005; Donos et al., 2003; Heijl et al, 1997). At least some of these discrepancies may be due to qualitative as well as quantitative differences in the multiple components between EMD and EMP, and several attempts have therefore been made to fractionate freshly isolated non-heat treated porcine EMP, in order to delineate the putative osteomodulatory components (Suzuki et al., 2005; Johnson et al., 2009). However, such partially purified preparations often contain growth factors such as BMP-2 and TGF-β1 that are likely to have pronounced effects on bone cell activity (Suzuki et al., 2005; Johnson et al., 2009; Nagano et al., 2006). In addition, these fractions have also been reported to contain components of differing molecular sizes, including low-molecular (< 6 kDa) weight proteins (Suzuki et al., 2005; Johnson et al., 2009; Nagano et al., 2006).

The present study has used two fractions of EMD (Fraction C and Fraction A) that have recently been isolated from a commercially available heat-treated extract of EMP (Emdogain®; EMD, Institut Straumann, Basel, Switzerland) using industrial scale protein fractionation methodologies (Mumulidu et al., 2007). The results obtained here demonstrated that 100 μg/ml of Fraction C, which comprises < 6 kDa EMD proteins, mainly TRAP, strongly suppressed the mineralization of PDL as well as other bone-forming cells, including osteogenic precursor/stem cell clone 7, AB cells and BMSC. Although Fraction C did not have any apparent effect on the early and intermediate osteogenic markers Runx2 and OP, respectively, the late markers OC, BSP and ALP were strongly inhibited by this fraction. In contrast, the presence of Fraction A, which comprises > 6 kDa EMD proteins and is devoid of Fraction C components, strongly stimulated the expression of early, intermediate and late osteogenic marker genes and also the terminal differentiation of the bone-forming cells. Thus, the variable outcomes of osteogenic differentiation *in vitro* and bone regeneration *in vivo* observed in the presence of non-fractionated EMD (Donos et al, 2005; Donos et al., 2003;

Heijl et al, 1997; Gestrelius et al., 1997; Van der pauw et al. 2000; Nagano et al. 2004; Hakki et al., 2001) may reflect at least partly differences in the relative proportions of these two fractions between different preparations of EMD and EMP.

It has previously been shown that the BMP-2 and TGF-β1 pathways are involved in the regulation of bone-forming cell activity (DaCosta et al., 2004; Miyazono, 2000; Alliston et al., 2001; Elford et al., 1987; Katagiri et al., 1994; Singhatanadgit et al., 2006). To determine whether EMD and the EMD Fractions acted via one or both of these signaling pathways, the present study used noggin, a BMP-2 pathway inhibitor, and UB-505124, a TGF-β1 pathway inhibitor, to examine if the presence of these inhibitors abrogated the osteogenic effects of EMD and the Fractions. The results showed that in the presence of these inhibitors the mineralization of the cells was further reduced, suggesting that Fraction C inhibits osteogenic differentiation at least partly via BMP-2- and TGF-β1-induced intracellular messengers, consistent with a previous report that, in mouse C2C12 cell line, low molecular weight components in EMP were able to suppress BMP-2 and TGF-β1-dependent osteogenesis (Suzuki et al., 2005). In addition, the results reported here demonstrate that Fraction C reduced the intracellular level of the activated BMP-2 transcription factor complex p-Smad1/5/8 in PDL cells and it also stimulated the expression of the Smad6 gene, which is known to act as a BMP-2 pathway negative regulator (Wu et al., 2004). Taken together these data indicate that Fraction C in EMD suppresses osteogenesis by up-regulating the inhibitory Smad6 that binds to and blocks the phosphorylation and subsequent nuclear translocation of the Smad1/5/8 complex.

In contrast to previous reports that BMP-2 and TGF-β1 growth factors may be present in a high molecular weight EMP fraction (e.g., 10-20 kDa) (Nagano et al., 2004; Suzuki et al., 2005; Johnson et al., 2009), in the present investigation it was observed that the stimulatory effects of Fraction A were unaffected by the BMP-2 and TGF-β1 inhibitors, suggesting that this Fraction (6 to 20 kDa components) is unlikely to contain BMP-2- and TGF-β1-like activities. Moreover, it further indicates that Fraction A stimulates bone via signaling pathway(s) independent of BMP-2 and TGF-β1, possibly such as those mediated by parathyroid hormone and the hedgehog and Wnt families (Williams et al., 2009; Wu et al., 2004).

Since TRAP and LRAP are major components of Fractions C and A, respectively, the present study also investigated the effects of chemically-synthesized TRAP and LRAP on

bone-forming cells. As with Fraction C, synthetic TRAP suppressed whereas, as with Fraction A, synthetic LRAP stimulated the osteogenic differentiation of the PDL cells. Although a previous report has suggested that the C-terminal amino acid sequence of LRAP might be responsible for the bioactivity of the parent protein (Veis et al., 2000), hitherto there has been no definitive evidence for the molecular identities of the active peptides. To delineate the specific sequences that are most likely to modulate osteogenic differentiation we compared the TRAP and LRAP amino acid sequences, which revealed that while the 33-amino acid sequence at the N-terminal of both the peptides are identical, the 12-amino acid (TCT) and the 23-amino acid (LCT) sequences at the C-terminals of TRAP and LRAP, respectively, are unique to each. These sequences were therefore chemically synthesized and examined for osteogenic activity, and found to inhibit and stimulate bone-forming cells, respectively. Moreover, protein homology analysis of the TRAP and LRAP amino acid sequences, using the National Centre for Biotechnology Information (NCBI) protein database and BLAST software (NCBI, Bethesda, MD), showed that these TCT and LCT amino acid sequences were the domains that were the most 'conserved' TRAP and LRAP sequences of > 100 different animal species, further indicating the possible physiological importance of these small unique C-terminal amino acid sequences in modulating the process of bone formation. These findings thus demonstrate, for the first time, that the C-terminal TCT and LCT peptide sequences exhibit the osteogenic bioactivities of the respective TRAP and LRAP amelogenin-derived parent peptides.

Although a number of techniques and materials are available for achieving bone regeneration, including the use of different types of bone graft as well as the FDA-approved BMP-2/INFUSE, their predictability and efficacy in terms of clinical outcome remain uncertain. The novel bioactive 23-amino acid LCT amelogenin sequence identified here can be produced synthetically in large quantities and with high purity, and may have the potential to significantly improve therapeutic options for periodontal disease, while the novel 12-amino acid TCT peptide might prove to be a valuable tool for limiting pathological bone cell growth.

In conclusion, two main fractions of EMD, Fraction C and Fraction A, which contain the amelogenin isoforms TRAP and LRAP, respectively, elicited differential bone-forming responses *in vitro*. Furthermore, while naturally occurring Fraction C and the synthetically produced TRAP and its unique C-terminal TCT sequence all strongly suppressed bone-forming cells, the naturally-occurring Fraction A and the synthetically produced LRAP and its

unique C-terminal LCT sequence strongly induced terminal differentiation of bone-forming cells.

Chapter 5

5.1. Introduction

A number of reports, including the present (Chapter 3), have shown that despite the absence of adipocytes in PDL tissue at least some cells in adult human PDL are nevertheless capable of adipogenic differentiation (Amin et al., 2011; Huang et al., 2009; Singhatanadgit et al., 2009; Kawanabe et al., 2010; Xu et al., 2009; Fujii et al., 2008). PDL also comprises a cell population capable of undergoing chondrogenesis *in vitro*, even though the maxilla and mandibular bones of the facial skeleton are considered to develop from MSC directly via intramembranous ossification (Chapter 1). Chondrogenic precursors are thus also unlikely to be present in the periodontium or to be involved in periodontal regeneration (Lee et al., 2001; Huang et al., 2009; Singhatanadgit et al., 2009; Kawanabe et al., 2010; Xu et al., 2009; Fujii et al., 2008).

Adipogenic and chondrogenic differentiation are two major pathways of mesenchymal differentiation and consequently widely studied in delineating mesenchymal stem cell-like populations. In addition, previous studies have shown that EMD can modulate these pathways in the mouse mesenchymal C2C12 and C3H10T1/2 and chondrogenic ATDC5 cell lines *in vitro* (Narukawa et al., 2006; Narukawa et al., 2007; Ohyama et al., 2002). Although EMD appeared to inhibit adipogenic differentiation and stimulate chondrogenic differentiation of these cells (Narukawa et al., 2006; Narukawa et al., 2007; Ohyama et al., 2002), it is not yet known which component(s) in EMD exhibit these (differential) modulatory activities. The present study therefore examined the effects of the various EMD components on the adipogenic and chondrogenic capabilities of PDL cells *in vitro*.

5.2. Materials and methods

PDL cell culture and RT-PCR were performed as described in Chapter 2; additional methods used in this chapter are described below.

5.2.1. Treatment of cells with EMD and the EMD Fractions

EMD, Fraction A and Fraction C were diluted in 0.1% acetic acid and added directly to the cells when they reached approximately 90% confluence. EMD and Fraction A were used at a final concentration of 100 μg/ml, whereas Fraction C was used at a final concentration of 10 μg/ml throughout the experiments, unless otherwise stated. These concentrations were determined from the dose effects of EMD, Fraction A and Fraction C on terminal PDL cell adipogenesis and chondrogenesis *in vitro*, as described below.

5.2.2. Effects of EMD and the EMD Fractions on adipogenic differentiation

The cells were cultured in GM and then adipogenic medium (AM) added, consisting of GM supplemented with 0.5 μM 3-isobutyl-1-methylxanthine, 50 μM indomethacine and 10 nM dexamethasone (all from Sigma) in the absence (control sample) and presence (test samples) of 1, 3, 10, 30 and 100 μg/ml of EMD, Fraction A and Fraction C to assess the effects of different doses on adipogenic differentiation, as follows (Amin et al., 2011). After 5 weeks, the cell monolayers were fixed with 10% formalin for 30 min, incubated with 60% propan-2-ol for 5 min and then stained with 0.2% Oil Red O in propan-2-ol for 5 min. After washing with tap water, the samples were counterstained with Harris Hematoxylin. For quantification of Oil Red O, the samples (without counter staining) were extracted into 100% propan-2-ol and the absorbance measured at 490 nm (A_{490}).

Total RNA was extracted from replicate cultures after 3 and 5 weeks for PCR analysis of the early adipogenic marker PPARγ2 (week 3) and the late marker LPL (week 5), as described in Chapter 2.

5.2.3. Effects of EMD and the EMD Fractions on chondrogenic differentiation

The PDL cells were trypsinized and $2.5x10^5$ cells suspended in 0.5 ml of serum-free chondrogenic medium (CM) (Lonza, Wokingham, UK) (low glucose-DMEM containing

insulin, transferrin, selenious acid, bovine serum albumin, linoleic acid, dexamethasone, ascorbate-phosphate, proline, pyruvate and TGF-β3) and placed in 15 ml conical polypropylene centrifuge tubes (Nunc). After centrifugation at 200g for 5 min, the pellets were incubated in CM in the absence (control sample) and presence (test sample) of 1, 3, 10, 30 and 100 μg/ml of EMD and the EMD Fractions at 37°C for 3 weeks, with caps loosened to allow gas exchange, then Alcian blue staining for acid mucopolysaccharides and glycosaminoglycans was also carried out to assess terminal chondrogenic differentiation as follows (Amin et l., 2011). Cell pellets were fixed in 10% formalin at 4˚C for 24 h, dehydrated in an ascending series of ethanol and embedded in paraffin. Sections (3 μm) were cut, stained with 1% Alcian blue (pH 2.5) (Sigma) for 5 min. The deposition of mucopolysaccharides and glycosaminoglycans was visualized as blue staining of the extracellular matrix. Nuclei were stained purple using Harris hematoxylin. Total RNA was extracted from the replicate cultures for PCR analysis of the early chondrogenic marker gene Sox-9 (after 1 week) and the late markers aggrecan and Col2a1 (after 2 weeks).

5.2.5. Statistical analysis

The RT-PCR data are presented as the mean of the triplicate measurements of cells cultured in AM or CM in the presence of EMD and the EMD Fractions compared with that of cell cultured in AM or CM alone (defined as 1.0). One-way ANOVA followed by post-hoc Bonferroni test (for multiple comparison) was used for statistical analysis (SPSS 12.0 software, Chicago, IL).

5. 3. Results

5.3.1. Dose-effects of EMD and the EMD Fractions on PDL cell adipogenesis

It was shown in Chapter 3 that PDL cells cultured in AM alone produced Oil red O- positive lipid-like droplets and that the Oil red O staining intensity was significantly higher in cells cultured in AM alone (1.3) compared with the cells cultured in non-differentiation-inducing GM (0.5). To examine the effects of EMD and the EMD Fractions on terminal adipogenesis, the PDL cells were cultured in AM in the absence and presence of varying concentrations (from 1-100 μg/ml) of EMD, Fraction A and Fraction C for 5 weeks, stained with the Oil red O and the Oil red O staining intensities measured as described in Section 5.2. The results in Figure 5.1 (i, ii) show that the addition of EMD, Fraction A and Fraction C at all concentrations (1 to 100 μg/ml) significantly reduced the formation of Oil red O-positive lipid-like droplets and the corresponding staining intensities of the PDL cells compared with the control cells cultured in AM alone ($p<0.05$). It is notable that the staining intensities of the cells cultured in the presence of EMD and the EMD Fractions were similar to that of the cells cultured in non-differentiation-inducing GM alone.

Moreover, representative PCR bands in Figure 5.2 (i) show that the cells cultured in AM with EMD and the EMD Fractions (all at 100 μg/ml) also expressed significantly reduced levels of the early and late adipogenic genes PPARγ2 and LPL, respectively, compared with cells cultured in AM alone ($p<0.05$) (Fig. 5.2 (ii)). These results thus suggest that EMD, Fraction A and Fraction C all suppress adipogenic differentiation of the PDL cells.

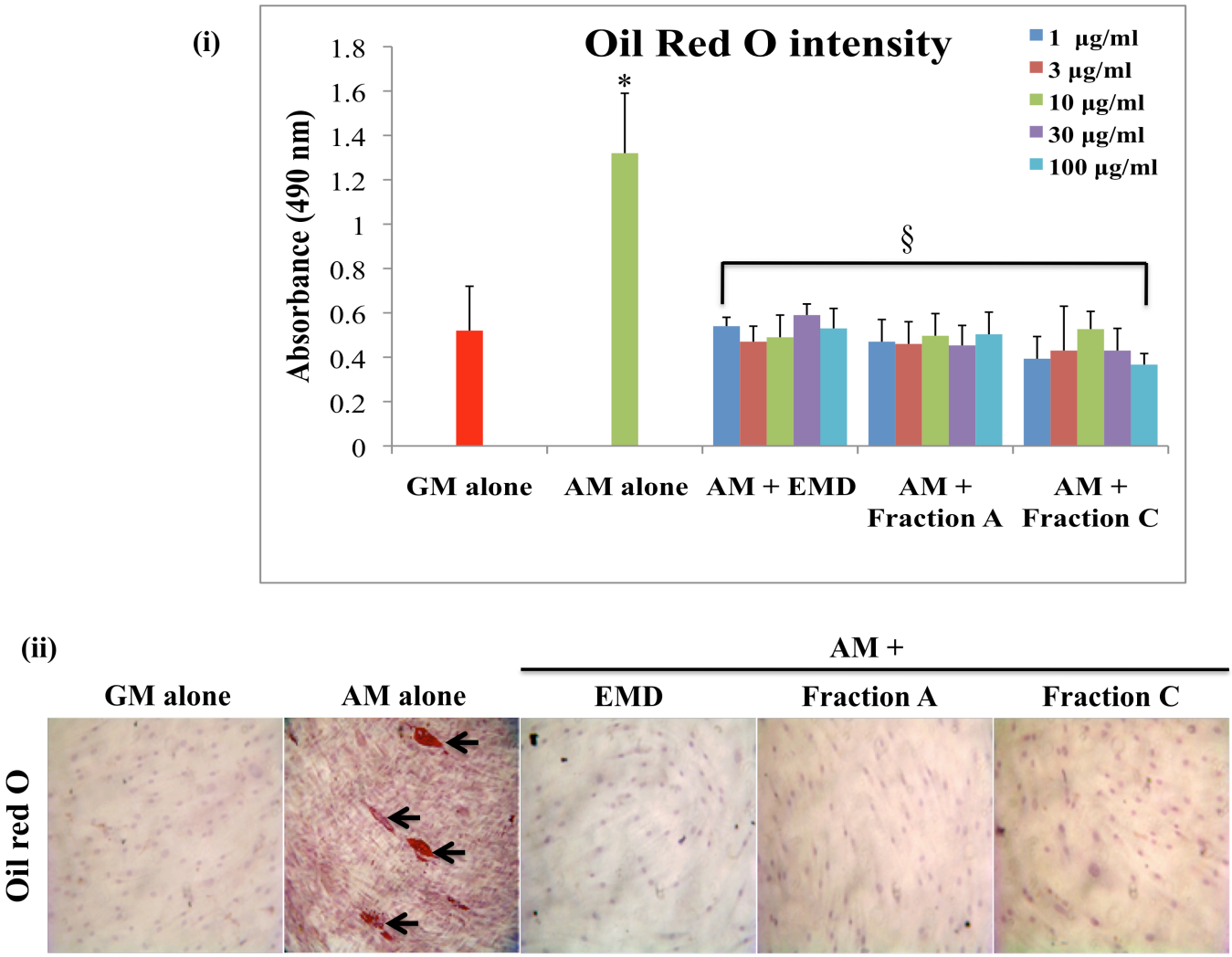


*Figure 5.1. Effects of varying concentrations of EMD, Fraction A and Fraction C on terminal adipogenic differentiation of PDL cells. (i) Oil red O staining intensity of the PDL cells cultured in AM in the presence of 1, 3, 10, 30 and 100 µg/ml EMD, Fraction A and Fraction C for 5 weeks. The values are the mean ±SE of triplicate measurements *Indicates statistically significant difference compared with GM alone ($p<0.05$); §Indicates statistically significant difference compared with AM alone ($p<0.05$). (ii) Representative Oil red O staining of PDL cells cultured in AM in the presence of EMD and the EMD Fractions for 5 weeks. Nuclei are counter-stained purple with Harris Hematoxylin, and the back arrows show lipid-like droplets stained red with Oil red O. Magnification x20.*

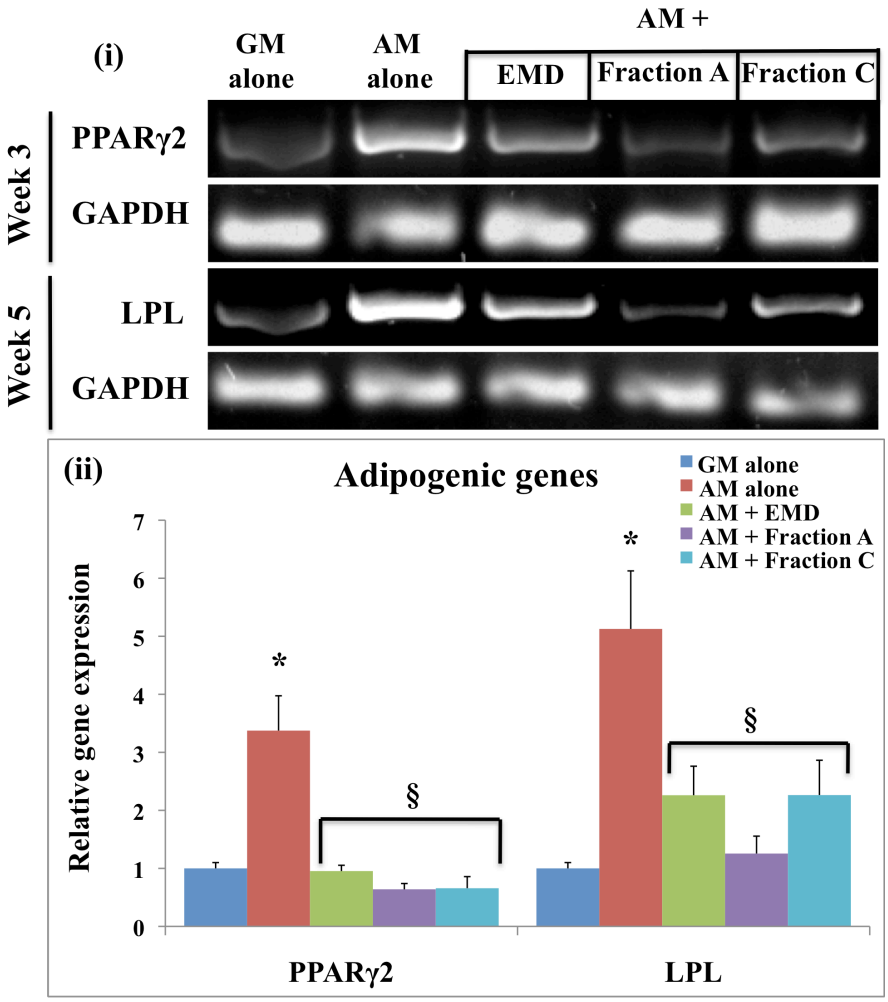


*Figure 5.2. Effects of EMD and the EMD Fractions on adipogenic genes of PDL cells. (i) A representative RT-PCR gel showing the expression of the adipogenic genes PPARγ2 and LPL by PDL cells cultured for 3 and 5 weeks, respectively, in AM in the presence of EMD, Fraction A and Fraction C. (ii) The values are the changes in PCR product band intensity relative to GAPDH of cells cultured in the presence of EMD and the EMD Fractions compared with AM alone, defined as 1.0. The values are the means ±SE of triplicate measurements. *Indicates statistically significant difference compared with GM alone ($p<0.05$); §Indicates statistically significant difference compared with AM alone.*

5.3.2. Dose-effects of EMD and the EMD Fractions on PDL cell chondrogenesis

It was shown in Chapter 3 that under culture conditions that enable chondrogenic differentiation, PDL cells in pellet cultures were able to produce Alcian blue-positive acid mucopolysaccharide and glycosaminoglycan-rich ECM characteristic of terminal chondrogenic differentiation *in vitro*, whereas Alcian blue-positive ECM was not detected in cultures incubated in non-selective GM (Fig. 5.3 (i)).

To examine the effects of EMD and the EMD Fractions on PDL cell chondrogenesis, cell pellets were cultured in CM in the presence of EMD, Fraction A and Fraction C (from 1-100 μg/ml) for 3 weeks, sectioned and stained with Alcian blue, as described in Section 5.2. The results in Figure 5.3 (ii) show that increasing concentrations of EMD progressively stimulated mucopolysaccharide and glycosaminoglycan production. Thus, strong Alcian blue staining of the ECM was observed in the presence of 100 μg/ml of EMD, while at lower concentrations of EMD (between 1 and 30 μg/ml) weaker staining was observed. In marked contrast, little if any Alcian blue-positive ECM was observed when cell pellets were cultured in the presence of Fraction A (from 1-100 μg/ml) (Fig. 5.3 (ii)). Notably, increasing concentrations of Fraction C were, like EMD, also found to have an apparent stimulatory effect on PDL cell chondrogenesis. Thus, while 1 and 3 μg/ml of Fraction C had little effect, 10 and 30 μg/ml appeared to produce substantial amounts of Alcian blue-positive ECM, which did not increase further at 100 μg/ml. These results indicate that 100 μg/ml of EMD and 10-30 μg/ml of Fraction C had the maximum stimulatory effect, whereas all the concentrations of Fraction A tested in the present study appeared to be inhibitory to terminal chondrogenic differentiation of the PDL cells.

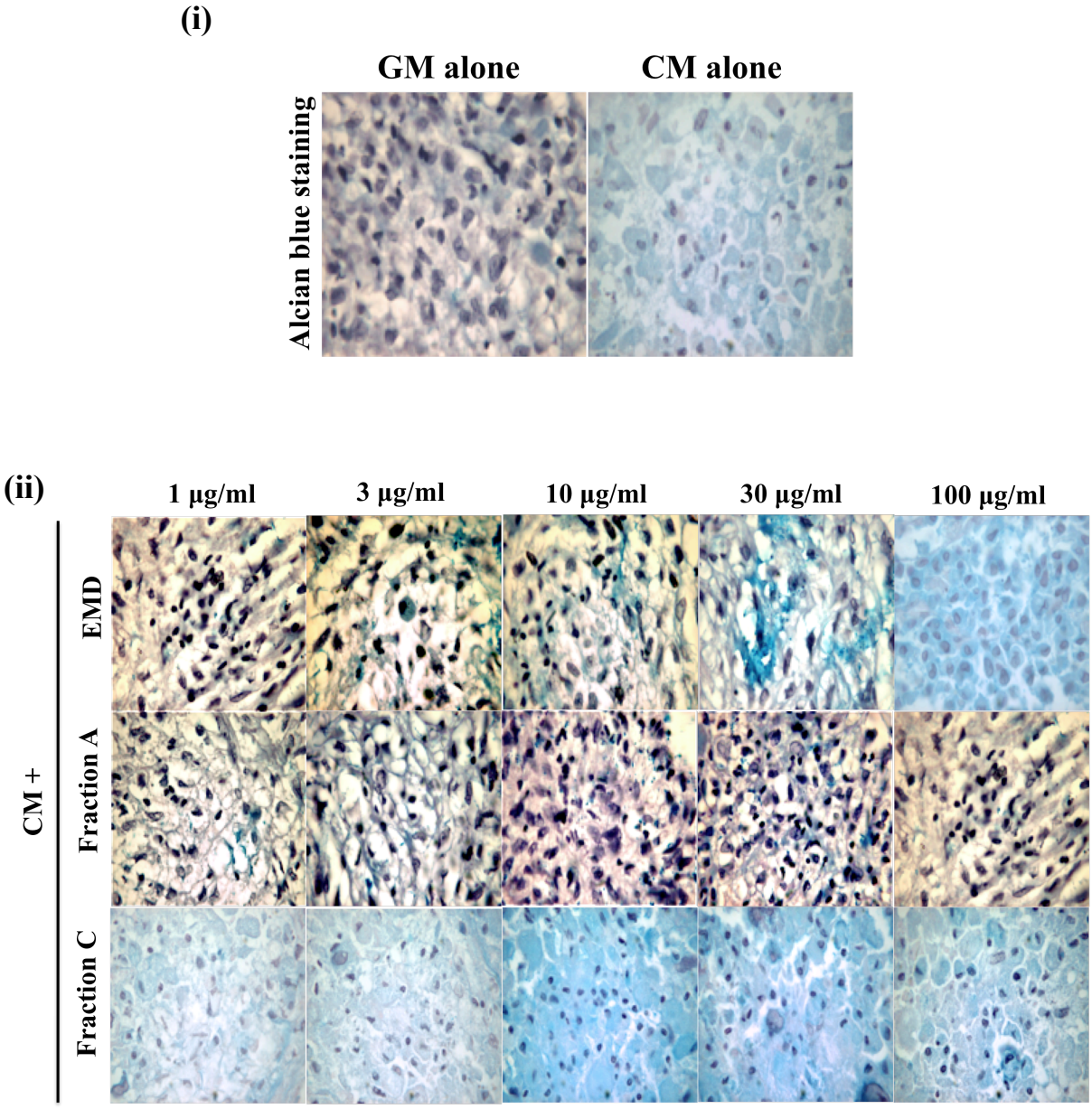


Figure 5.3. Effects of varying concentrations of EMD and the EMD Fractions on terminal chondrogenic differentiation. Alcian blue staining of paraffin sections (3 µm) prepared from pellet cultures maintained for 3 weeks in (i) GM and CM alone and (ii) in CM with 1, 3, 10, 30 and 100 µg/ml EMD, Fraction A and Fraction C. Nuclei are counter-stained purple with Harris hematoxylin and the Alcian blue stained ECM was visualized blue, which is particularly apparent in CM + EMD and CM + Fraction C. Magnification x50.

It was shown in Chapter 3 and here that under chondrogenic conditions, PDL cells in pellet cultures expressed higher levels of the early chondrogenic transcription factor Sox-9 (3.2-fold) and the late genes Aggrecan (1.7-fold) and Col2a1 (1.9-fold) compared with pellets cultures in GM alone ($p<0.05$) (Fig. 5.4 (i, ii)). However, representative PCR bands show that the presence of EMD, Fraction A and Fraction C in CM did not cause any significant change in the Sox-9 gene compared with the cells cultured in CM alone (Fig. 5.4 (i, ii)). In contrast, the late chondrogenic matrix gene Aggrecan and Col2a1 were both markedly stimulated by EMD (3.8- and 4.0-fold) and by Fraction C (9.4- and -10.5-fold) compared with cells cultured in CM alone ($p<0.05$). In contrast, as with terminal chondrogenic differentiation, Fraction A significantly suppressed the Aggrecan and Col2a1 genes compared with cells cultured in CM alone ($p<0.05$). These results thus suggest that EMD and Fraction C strongly stimulate, whereas Fraction A inhibits, PDL cell chondrogenesis.

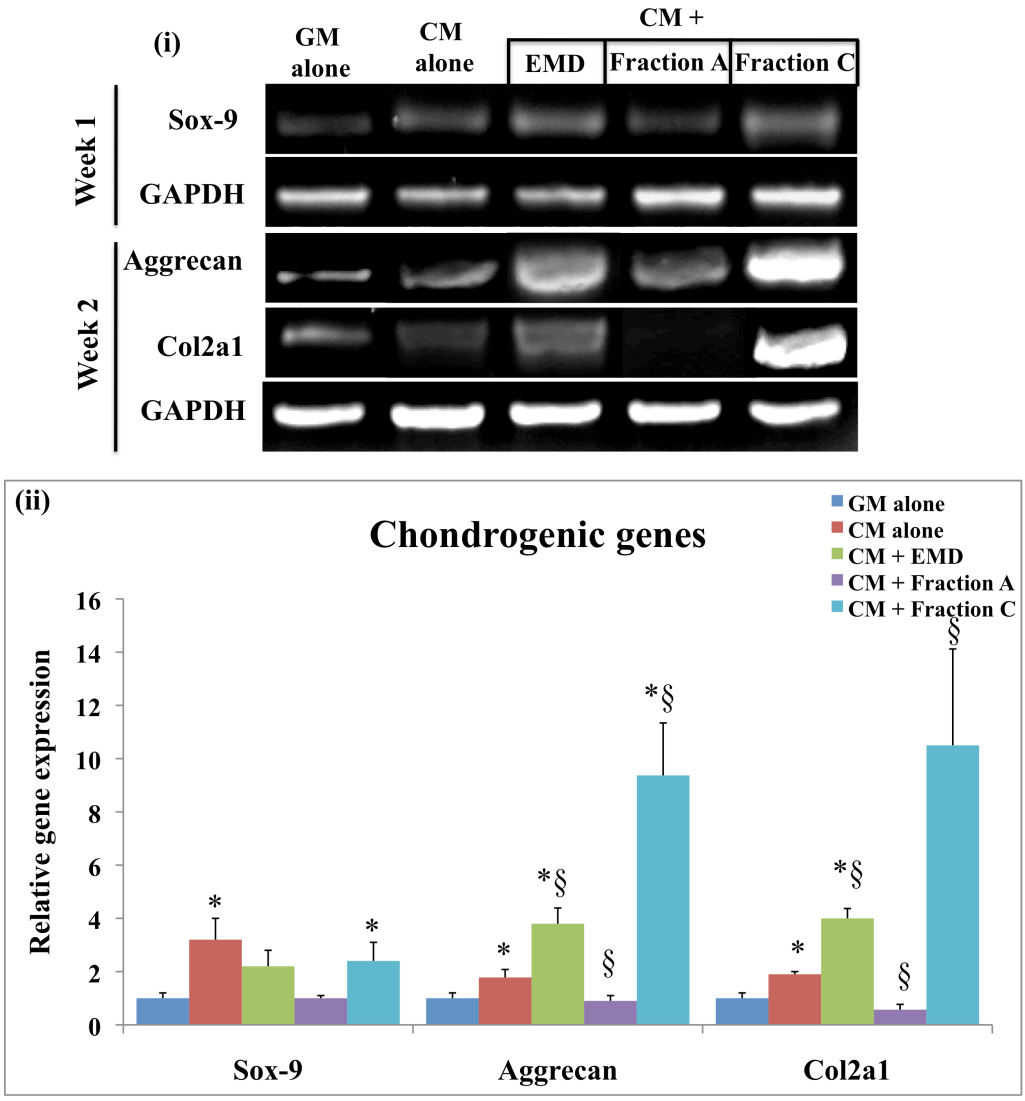


*Figure 5.4. Effects of EMD and the EMD Fractions on chondrogenic gene expression by PDL cells. (i) A representative RT-PCR gel showing the expression of Sox-9 (week 1) and the late Aggrecan and Col2a1 (week 2) genes by PDL cells cultured in CM in the presence of EMD, Fraction A and Fraction C. (ii) The values are the changes in PCR product band intensity relative to GAPDH obtained in CM in the presence of EMD and the EMD Fractions compared with CM alone, defined as 1.0. The values are the means ±SE of triplicate measurements. *Indicates statistically significant difference compared with GM alone ($p<0.05$);§Indicates statistically significant difference compared with CM alone ($p<0.05$).*

5.4. Discussion

Despite the histological absence of adipocytes and chondrocytes from PDL tissue, PDL cells have been shown (Chapter 3; Amin et al., 2011; Singhatanadgit et al., 2009; Tomokiyo et al., 2007; Xu et al., 2009; Cheng et al., 2009) to be able to differentiate into functional adipocytes, cells capable of producing lipid-like droplets, and chondrocytes, cells with the ability to produce mucopolysaccharide- and glycosaminoglycan-rich ECM. It is thus likely that the PDL contains a precursor/stem cell-like population capable of undergoing these key mesenchymal differentiation pathways, in addition to osteogenesis as shown in Chapter 3, and the effects of EMD on these pathways have previously been investigated in order to understand the regulation of mesenchymal stem cells, including bone precursors (Narukawa et al., 2006; Narukawa et al., 2007; Ohyama et al., 2002). Thus EMD has previously been found to modulate adipogenic and chondrogenic differentiation of several different mouse mesenchymal and chondrogenic cell lines (Narukawa et al., 2006; Narukawa et al., 2007; Ohyama et al., 2002), and the present study investigated whether components in EMD were able to regulate the adipogenic and chondrogenic differentiation pathways in PDL cells.

The present results showed that while culture in AM alone up-regulated both the early PPARγ2 and the late LPL gene, and produced lipid-like droplets which stained positive for oil red O, all concentrations of EMD and the EMD Fractions tested here strongly suppressed terminal adipogenic differentiation and markedly inhibited adipogenic genes. Similarly, although the Fractions of EMD were not used, EMD was shown to suppress adipogenic marker genes PPARγ2 and LPL and up-regulate osteogenic markers ALP and osteocalcin of the mouse mesenchymal cell line C2C12 (Ohyama et al., 2002). Although the exact reason for this is not clear, it possible that the inhibitory activity of EMD and the EMD Fractions may at least partly be due to the modulation of the Runx2-PPARγ2 ‘switch’, a mesenchymal cell differentiation regulatory mechanism in which the adipogenic PPARγ2 gene is down-regulated while the osteogenic transcription factor Runx2 is concomitantly up-regulated (Takada et al., 2009), consistent with the observation that EMD- and the EMD Fraction A down-regulated PPARγ2 and up-regulated Runx2 (Chapter 4). Moreover, EMD has been shown to stimulate osteogenesis via BMP and TGFβ signal transduction pathways and these signaling pathways have previously been shown to inhibit adipogenesis (Katagiri et al., 1994;

Suzuki et al., 2005; Van der Pauw et al., 2000). These results thus demonstrate that the EMD and the EMD components strongly suppress adipogenic differentiation of PDL cells.

As shown in Chapter 3, under CM PDL cell pellets expressed chondrogenic genes Sox-9 and aggrecan and stained for Alcian blue-positive mucopolysaccharide and glycosaminoglycan-rich ECM. The addition of EMD and Fraction C further increased, whereas Fraction A decreased, overall staining of mucopolysaccharide and glycosaminoglycan in ECM and the expression of the late chondrogenic genes Aggrecan and Col2a1 expression of the PDL cells. In contrast to a previous report (Narukawa et al., 2007) in which EMD was found to stimulate Sox-9 expression of chondrogenic ATDC5 cell line, the present study showed that EMD and the EMD Fractions did not affect the Sox-9 gene of PDL cells. This may be due at least partly to the use of non-heat treated EMD preparation in the previous study, which was found to have IGF and TGFβ-like growth factors, and also to differences in the types of cell. These findings thus suggest the possibility that while culture in CM resulted in stimulation of the key chondrogenic transcription factor Sox-9, the presence of EMD and Fraction C components in EMD up-regulated the late phase of chondrogenic differentiation (production of proteoglycan-rich ECM) by PDL cells cultured in pellets *in vitro*.

These results demonstrate that although EMD and the EMD Fractions suppressed PDL adipogenesis, Fraction A and Fraction C exhibited differential effects on chondrogenesis of PDL cells, as found with osteogenesis (Chapter 4).

Chapter 6

6.1. Introduction

The PDL that supports the teeth is comprised primarily of fibroblasts, osteoblasts and osteoclasts and has recently been reported to also contain a progenitor/stem cell-like population that can undergo multi-lineage differentiation (Amin et al., 2011; Cheng et al., 2009; Huang et al., 2008; Nagatomo et al., 2006; Singhatanadgit et al., 2009; Tomokiyo et al., 2007; Xu et al., 2009). The homeostasis, repair and regeneration of PDL tissue are considered to be dependent on such progenitor cells and also the appropriate biological mediators and a sufficient blood supply (Cheng et al., 2009; Cochran et al., 1999; Huang et al., 2008; Molloy et al., 2003; Taba et al., 2005). Thus, growth factors, cytokines and morphogens have been shown to modulate the proliferation and differentiation of PDL cells and the production of extracellular matrix (ECM) *in vitro* (Cheng et al. 2009; Cochran et al., 1999; Huang et al., 2008; Karring et al., 1993; Messenger et al., 2007; Molloy et al., 2003), while new vascular networks formed by progenitor cells ensure the supply of sufficient blood for the repair/wound healing of damaged PDL and the regeneration of healthy new tissue *in vivo* (Brey et al., 2005; Taba et al., 2005).

It is now recognized that BV formation, the complex process of neovasculogenesis, comprises both vasculogenic and angiogenic differentiation during adult wound healing as well as in developing microenvironments (Brey et al., 2005; Flamme et al., 1997). The former process, vasculogenesis, the differentiation of progenitor/stem cells into endothelial cells (Brey et al., 2005; Flamme et al., 1997; Demir et al., 2007) and the latter, angiogenesis, the development of an organized network of tubular structures originating from endothelial precursors (Brey et al., 2005; D'Amore et al., 1987; Flamme et al., 1997), are regulated *in vitro* and *in vivo* by a number of biological mediators including vascular endothelial growth factor (VEGF), platelet-derived growth factor, insulin-like growth factor (IGF), transforming growth factor-beta (TGF-β), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Brey et al., 2005; Cochran et al., 1999; Lynch et al., 1991; Molloy et al., 2003; Nomi et al., 2002; Pandya et al., 2006) and also by enamel matrix proteins (EMP) (Bertl et al., 2009; Johnson et al., 2009; Kauvar et al., 2010; Messenger et al., 2007; Schlueter et al., 2007).

EMP is a heterogeneous mixture of components consisting primarily of peptide isoforms derived from the full-length amelogenin gene transcript and is secreted during tooth development (Fincham et al., 1993). Crude preparation (non-heat treated) of EMP and commercially prepared heat-treated EMD has widely been used clinically for regenerating periodontal tissues, including PDL, alveolar bone and cementum, a mineralized tissue found uniquely in the periodontium (Hammarstrom et al., 1997; Cochran et al., 1999; Donos et al., 2003). Several clinical studies carried out to evaluate the effects of EMP and EMD on periodontal soft tissue wound healing have led to a number of discrepancies (Wennstrom et al., 2002; Hagennars et al., 2004). For example, periodontal wounds treated topically with EMP exhibited rapid and complete healing, compared with the control sites that were not treated with EMP which exhibited only partial healing (Wennstrom et al., 2002). In contrast, it was shown that the test patient group treated with EMD exhibited only partial healing, similar to the control group that did not receive EMD (Hagennars et al., 2004). The reason(s) for this discrepancy are not known, but it may be at least partly due to lack of sufficient blood supply, i.e. differences in BV formation activity/neovasculogenic activity.

A number of attempts have therefore been made *in vitro* to understand these apparent clinical discrepancies by examining the effects of EMD on angiogenic differentiation *in vitro*. However, such studies have been limited because some have used freshly isolated and non-heat treated EMP while others have used commercially prepared and heat-treated EMD (Johnson et al., 2009; Yuan et al., 2003; Schlueter et al., 2007; Bertl et al., 2009; Thoma et al., 2010). For example, while freshly extracted EMP has been found to strongly stimulate migration/chemotaxis and formation of a tubular network of human microvascular endothelial cells (HMVEC) *in vitro* (Johnson et al., 2009; Yuan et al., 2003), both Schlueter (2007) and Bertl (2009) showed that commercially prepared EMD did not induce HMVEC migration/chemotaxis in an *in vitro* monolayer cell wound healing assay. Since the components of EMP preparations are likely to vary qualitatively and quantitatively (detailed description in **Section 9.1.2**), a number of attempts have also been made to delineate the specific protein fractions in freshly isolated (and non-heat-treated) EMP and commercial preparation EMD that have angiogenic activity (Johnson et al., 2009; Thoma et al., 2010). For example, Johnson (2009) examined the effects of EMP fractions on HMVEC angiogenesis *in vitro* and observed that both low and high molecular weight fractions stimulate chemotaxis and tube formation of HMVEC, but the EMP fractions used contained proteins of differing sizes, with all the fractions containing some low molecular weight peptides. In addition,

Thoma (2011) examined the effects of EMD in a murine angiogenesis model *in vivo* and reported that both the low (< 6 KDa) and high (< 15 kDa) molecular weight fractions of heat-treated EMD exhibited angiogenic activity, but could not identify specific component(s). Again, low molecular weight proteins were found to be present in all the fractions isolated from EMD (Thoma et al., 2011) and indicating that discrepancies in the effects of EMP and EMD on angiogenesis *in vitro* and *in vivo* could be associated with the use of heterogeneous fractions whose components could have differential effects on angiogenesis and use of differing endothelial cell culture conditions including culture media and length of time of EMP and EMD treatment.

As described in previous Chapters (Chapters 4 and 5), two sub-fractions of the commercially available EMD, Fraction C and Fraction A, have been obtained by industrial scale protein fractionation methodologies (Mumulidu et al., 2007), with Fraction C comprising < 6 kDa peptides (mainly a 5.3 kDa tyrosine-rich amelogenin peptide (TRAP)) and Fraction A containing a mixture of > 6 kDa peptides including a leucine-rich amelogenin peptide (LRAP), sheathlin proteins and the full-length amelogenin protein (Mumulidu et al., 2007). The latter components have been shown to have a role in the formation of hard tissue, including bone and cementum (Kanazashi et al., 2006; Warotayanont et al., 2008; Chapter 4), but neither Fraction C nor Fraction A have hitherto been examined to determine whether they affect neovasculogenesis. It was shown in Chapter 3 that PDL cells have the capacity to undergo vasculogenic differentiation when cultured in endothelial medium *in vitro*. The present study has therefore examined the response of PDL cells to EMD, Fraction A, Fraction C and chemically synthesized TRAP, the 5.3 kDa peptide that is the main component of low molecular weight Fraction C, when cultured under conditions that facilitate vasculogenic differentiation *in vitro*.

In Chapter 3 it was also shown that the PDL cells when cultured on gel of a basement membrane proteins (laminin, collagen type IV, heparan sulfate proteoglycan, entactin and nidogen) were able to undergo angiogenic differentiation and form tubular-like structures with minimal sprouting. Thus, the present study examined the effects of EMD components on PDL cell angiogenesis *in vitro*, including PDL cell chemotaxis and the ability to form such BV-like structures. In addition, the chick embryo chorio-allantoic membrane (CAM), an extra-embryonic highly vascularized membrane that serves as a transient gas exchange surface similar to the lungs (New, 1955; Auerbach et al., 2003; Chapman et al., 2001), was used in the present study to determine the effects of EMD components on angiogenesis *ex*

vivo. The CAM model has been used extensively to test angiogenic factors (banzoxazine, VEGF, bFGF, EGF) due to the readily accessible vascularized CAM surface that enables precise monitoring and the reproducibility of the experimental system (Dong et al., 2010; Auerbach et al., 2003; Chapman et al., 2001; Kauvar et al., 2010).

6.2. Materials and methods

PDL cells were used to examine the effects of EMD components on vasculogenic and angiogenic differentiation (Amin et al., 2011; Chapter 3). Human umbilical vein endothelial cells (HUVEC) (Lonza, Slough, UK) were used as the positive control for angiogenic differentiation, as they have previously been shown to be capable of angiogenic differentiation (Dong et al., 2010; Schlueter et al., 2007; Johnson et al., 2009). PDL and EC culture and RT-PCR were performed as described in Chapter 2; additional methods used in this chapter are described below.

6.2.1. Treatment of cells with EMD components

EMD and the EMD-derived < 6 kDa (Fraction C) and > 6 kDa (Fraction A) fractions were diluted in 0.1% acetic acid and a range of concentrations (1-100 μg/ml) added directly to the PDL cell cultures when they reached approximately 90% confluence. In addition, the chemically synthesized TRAP (sequence: NH_2-MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPYTSYGYEPMGGW–COOH; provided by Institut Straumann, Basel, Switzerland), the major component of Fraction C, was diluted in cell culture grade water (Thermo Scientific, Basingstoke, UK) and 30 μg/ml added directly to the cells when they reached approximately 90% confluence. The concentration of TRAP was determined from the dose effects of EMD and the EMD Fractions on terminal vasculogenesis of PDL cells *in vitro*, as described in Section 6.3.1.1.

6.2.2. Effects of EMD and the EMD Fractions on PDL cell vasculogenic differentiation

6.2.2.1. Immunocytochemical evidence of vasculogenesis

PDL cells were seeded into 24-well plates at a density of 2.5 x 10^4 cells/well, cultured in GM for 2-3 days and the medium replaced with endothelial basal medium-2 containing increasing concentrations of EMD components (1-100 μg/ml). EBM-2 supplemented with the endothelial growth factors (GF) VEGF (150 ng/ml) and EGF (50 ng/ml) (EBM-2 + GF) (Peprotech, London, UK), a medium which has previously been shown to induce murine and human mesenchymal stem cell (MSC) vasculogenesis *in vitro* (Wang et al., 2010; Gang et al., 2006), was used to facilitate vasculogenic differentiation, as previously described (Wang et al., 2010; Gang et al., 2006). The EBM-2, EBM-2 + EMD components and EBM-2 + GF

were changed every 3-4 days. After 5 weeks, immunostaining for the vasculogenic markers VE-cadherin, an EC adhesion protein, and von Willebrand factor (vWF), a blood glycoprotein localized in EC-specific vesicles, was also carried out on replicate cultures, as previously described (Amin et al., 2011). Briefly, cells were fixed in 4% paraformaldehyde (Merck, Poole, UK) for 15 min at room temperature (RT) and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% normal goat serum (NGS) in PBS for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti-VE-cadherin (Insight Biotechnology, London, UK) and anti-vWF (Abcam, Cambridge, UK) antibodies diluted 1:100 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. VE-cadherin- and vWF-positive cells were visualized by their green fluorescent staining. Nuclei were stained blue using Hoechst dye. The proportion of VE-cadherin and vWF-positive cells was determined by manual counting of 3 separate fields of each culture.

Total RNA was extracted from replicate cultures for conventional RT-PCR analysis of the VEGF receptor-1 (VEGFR1) and VEGFR2 (measured at week 1) genes involved at an early stage of VEGF-mediated vasculogenic differentiation. The Tie-1 and Tie-2 genes involved in an angiopoietin-mediated late stage of vasculogenesis were measured after 2 weeks, and the VE-cadherin gene involved in producing EC adhesion protein at a late stage of vasculogenesis was measured at week 3, as described in Chapter 2. Since mRNA transcripts are expressed much earlier than the corresponding proteins, the VE-cadherin gene was measured at week 3 and immunostaining of VE-cadherin protein was carried out at week 5.

6.2.2.2. Functional assay of vasculogenic differentiation by Flow Cytometry (FCM)

Receptor-mediated endocytosis of low-density lipoprotein (LDL), involved in the transport of triglycerides and cholesterol into the blood, is a key functional indicator of terminal vasculogenic differentiation (Anderson et al., 1977). To determine whether PDL cells exhibited this activity when cultured in differentiation medium with EMD component(s), PDL cells were cultured in 24-well plates in GM for 2-3 days and then replaced with EBM-2 alone, EBM-2 + EMD components (test sample) and EBM-2 + GF (positive control sample). After 5 weeks, cultures were incubated for 2 h in EBM-2 with 100 ng/ml Alexa Fluor labelled-acetylated-LDL (Invitrogen, Paisley, UK), fixed with 4% PFA for 15 min at RT and

nuclei stained using Hoechst dye. Flow cytometry (FCM) was also carried out on replicate cultures to measure the intracellular levels of Alexa Fluor labelled-acetylated-LDL, as previously described (Singhatanadgit et al., 2009). The cells were detached from the culture dish by incubating with trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 1 mM EDTA) (Gibco) for 5 min at 37^{o}C, centrifuged, fixed in 4% PFA and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. The cells were then centrifuged and re-suspended in 200 µl of PBS and the fluorescence intensity of 10,000 individual cells measured using a flow cytometer (FACScan; Becton-Dickinson, Cowley, UK). Data analysis was carried out using the WinMDI 2.8 software program.

6.2.3. Effects of EMD components on angiogenesis

6.2.3.1. Chemotaxis assay

One of the initial events of angiogenic differentiation *in vivo* is the chemotaxis/migration of endothelial precursors to the wound site followed by formation of angiogenic structures by the accumulated endothelial precursors (Bauer et al., 2005; Yuan et al., 2003). To examine the effect of EMD components on PDL cell chemotaxis, a 2-dimentional cell 'wound healing' migration assay was performed, as previously described (Bertl et al., 2009; Staton et al., 2009). Briefly, 2.5 x 10^4 EC/well were cultured in GM alone in a 24-well plate for 3-4 days or until confluent, after which a 'wound' was created *in vitro* by scraping off the cells using a pipette tip (200 µl) in a straight horizontal line (1 mm wound in width). The cultures were then washed with PBS and re-cultured with EBM-2 alone, EBM-2 + EMD components (test samples) and EBM-2 + GF (positive control samples) for 12 h. For analysis of cell migration (i.e., wound healing activity), microscopic images of the wound were taken immediately after scraping the cells (0 h) and after 6 and 12 h in the absence and presence of EMD components and GF. Cell migration was quantified by measuring the area of the wound (in pixel counts) using ImageJ software, using the following equation: area of wound (no cells) at 0 h minus area of wound at 6 or 12 h in the absence and presence of TRAP and GF. Full healing of a monolayer wound is the full closure of wounded area as a result of cell migration, while no healing is equivalent to zero (the cell-free wound area is the same at the 0 h test time period). Replicate experiments were also performed using HUVEC as a positive control.

6.2.3.2. Angiogenic structure formation *in vitro*

Angiogenic structure formation by endothelial precursors *in vitro* was performed using an angiogenesis assay kit, as described in Chapter 3 and also in previous reports (Millipore, Billerica, MA, USA) (Dong et al., 2010; Auerbach et al., 2003; Zhang et al., 2008). The assay comprises a gel of basement membrane proteins (laminin, collagen type IV, heparan sulfate proteoglycan, entactin and nidogen) on which the endothelial precursors are cultured and align to form polygonal tube-like structures in a multi-step process involving cell adhesion, migration, growth and differentiation (Bauer et al., 2005; Auerbach et al., 2003; Zhang et al., 2008). In this assay, 10^4 PDL cells were plated on gel-coated 96-well plates and cultured in the presence of EBM-2 alone, EBM-2 + EMD components (test samples) and EBM-2 + GF (positive control sample). After 5 and 15 h, digital images were obtained using bright-field microscopy. For quantitative analysis, branch points of polygonal tube-like structures in each culture condition were scored in 5 random fields, as described by the manufacturer of the kit and other reports (Dong et al., 2010; Schlueter et al., 2007). Similar experiments were also carried out using HUVEC as a positive control.

6.2.3.2. Chick chorioallantonic membrane (CAM) assay of angiogenesis *ex vivo*

The CAM is a specialized highly vascular extra-embryonic tissue formed during avian embryogenesis. The chick embryo CAM angiogenesis assay has been widely used to test angiogenic compounds including bFGF (Esch et al., 1985; Rabatti et al., 1995), VEGF (Wilting et al., 1992), EGF (Stewart et al., 1989), retinoids (Oikawa et al., 1989) and somatostatin (Woltering et al., 1991), mainly due to its readily accessible vascularized CAM, relatively rapid outcome and accessibility for monitoring BV formation over the course of the experiment (New, 1955; Auerbach et al., 2003; Chapman et al., 2001). The present study therefore used this model to examine the effects of EMD components on angiogenesis *ex vivo*. Fertilized White Leghorn chick eggs (Joice and Hill Poultry Ltd, Peterborough, UK) were incubated at 37^0C in 70-80% relative humidity in air for 3.5 days. The eggs were then carefully cracked open and the developing embryos transferred into 10 cm^2 petri dishes and re-cultured. On day 7, UV-sterilized filter disks (3 mm^2) were placed on the CAM where BV were visible and 15 µl of EMD components containing 1, 25, 50 and 100 µg/ml (test samples) added drop-wise onto the disks. Replicate eggs were treated with 15 µl of 100 µg/ml bovine serum albumin in cell culture grade water (Thermo Scientific) as control samples. The eggs

were then re-incubated for a further period of 3 days and the capillaries sprouted from the arteries of the CAM around the disks were photographed, and total number of capillaries which had sprouted were counted in each micrograph and analyzed using ImageJ software, as previously described (Borges et al., 2003; Dong et al., 2010).

6.2.4. Statistical analysis

The RT-PCR data are shown as the mean fold-change ± standard error (± SE) of three separate experiments compared with that of control cells cultured in EBM-2 alone (defined as 1.0). One-way ANOVA was used to assess statistically significant differences ($p<0.05$) followed by the post-hoc Bonferroni test ($p<0.05$) for multiple comparisons between the means (SPSS 11.0 software, Chicago, IL).

6.3. Results

6.3.1. Vasculogenic differentiation

6.3.1.1. Effects of increasing concentrations of EMD and the EMD Fractions on vasculogenic differentiation of PDL cells

Vasculogenic differentiation of progenitor/stem cells into EC is considered pivotal in new BV formation in embryological development and also in adult wound healing (Brey et al., 2005; Flamme et al., 1997). Vasculogenesis of adult cells has been examined here by culturing PDL cells in endothelial basal medium (EBM-2) for 5 weeks (Chapter 3). To examine whether EMD and the EMD Fractions modulate this process the PDL cells were cultured in EBM-2. The representative micrographs in Figure 6.1 show that when PDL cells were incubated in EBM-2 alone and EBM-2 + EMD, only few VE-cadherin immunostained cells were observed. However, in the presence of Fraction C (30 μg/ml) VE-cadherin-positive cells were more evident and the staining was found to be localized at the lateral borders of the cells (junctions), as previously reported using endothelial precursors derived from human umbilical cord (Alghisi et al., 2009; Muller et al., 2002) (Fig. 6.1). In contrast, in the presence of Fraction A there were no VE-cadherin-positive cells. The results in Table 5 show the effects of increasing concentrations (1-100 μg/ml) of EMD, Fraction C and Fraction A. 8.5±1.3% of PDL cells were found to stain positive for VE-cadherin when cultured in EBM-2 alone for 5 weeks, as previously noted in Chapter 3. When the cells were cultured in EBM-2 in the presence of 1, 3 and 10 μg/ml of EMD there was no increase in % of VE-cadherin-positive cells (9.2, 9.7 and 10.2% positive cells, respectively). However, in the presence of 30 μg/ml and 100 μg/ml of EMD, 13.0 and 15.4% of VE-cadherin-positive cells were observed, significantly higher than the cells cultured in EBM-2 alone (Table 5). Increasing concentrations of Fraction C markedly increased the number of VE-cadherin-expressing cells, with 30 μg/ml resulting in 29.2% positive cells, although the presence of 100 μg/ml of Fraction C did not further increase the relative proportion of positive cells (Table 5). It is notable that the % of VE-cadherin-positive cells in the presence of Fraction C (30 and 100 μg/ml) were significantly higher than the % of positive cells observed in the presence of EMD (30 and 100 μg/ml) (Table 5). These results thus indicate that Fraction C appears to actively promote vasculogenic differentiation and further experiments were therefore carried out (see **Section 6.3.1.2**, below) to clarify the activity of this Fraction by using the TRAP peptide, the

main component of Fraction C, which was obtained by chemical synthesis. Fraction A, which was found to suppress PDL cell vasculogenic differentiation was not further examined.

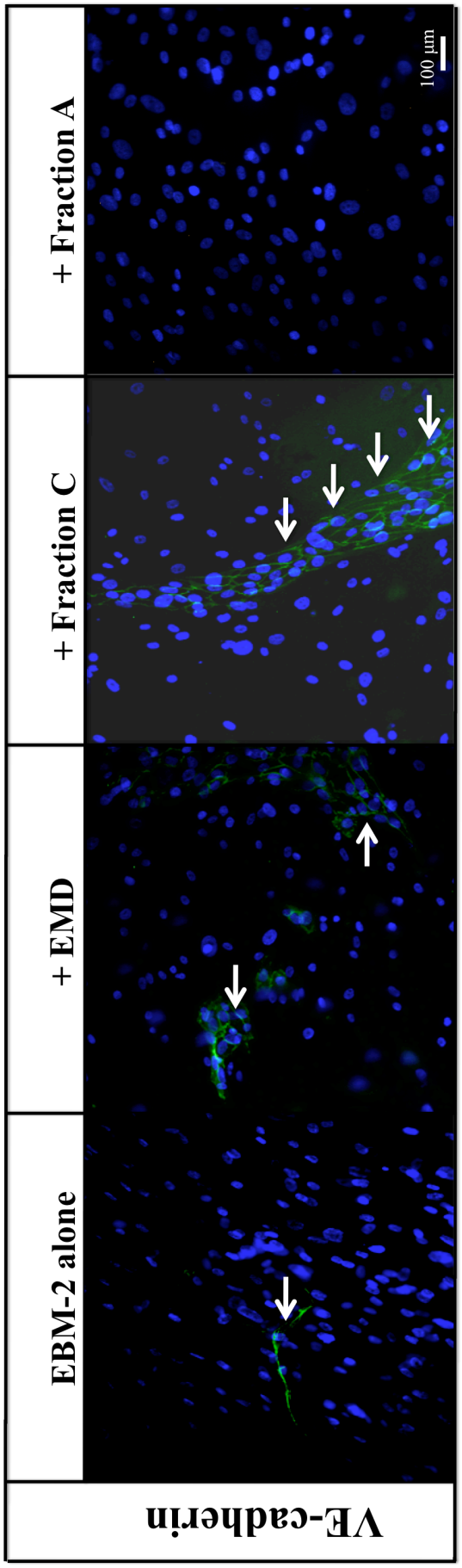


Figure 6.1. Representative micrographs of VE-cadherin immunostaining of PDL cells cultured in EBM-2 alone and EBM-2 + EMD (100 µg/ml), Fraction C (30 µg/ml) and Fraction A (100 µg/ml) for 5 weeks. The nuclei are stained blue with Hoechst dye, and the white arrows show the green fluorescence of VE-cadherin- positive cells.

Conc. (µg/ml)	VE-cadherin-positive cells (% of total cells)		
	EMD	**Fraction C**	**Fraction A**
1	9.2±1.6	9.3±1.9	3.1±1.4*
3	9.7±1.7	15.1±2.2*§	2.6±1.7*
10	10.2±1.8	20.7±3.0*§	1.1±0.8*
30	13.0±2.2*	29.2±3.4*§	0.0
100	15.4±1.2*	27.4±4.0*§	0.0

*Table 5. Effect of increasing concentrations (1-100 µg/ml) of EMD and the EMD Fractions on VE-cadherin staining of PDL cells cultured for 5 weeks in EBM-2. The numbers are the % of VE-cadherin-positive cells, as described in the Materials and methods. The values are the means ±SE of three measurements of three separate experiments. *Indicates significant difference compared with EBM-2 alone ($p<0.05$). §Indicates significantly higher than EMD ($p<0.05$).*

The control cultures (EBM-2 alone) had 8.5±1.3% VE-cadherin positive cells after 5 weeks

6.3.1.2. Effects of TRAP on vasculogenic differentiation

As noted above, Fraction C, which has previously been reported to contain the TRAP peptide (Mumulidu et al., 2007), was found here to markedly increase vasculogenic differentiation of PDL cells **(Section 6.3.1.1)**. The TRAP peptide was therefore obtained by chemical synthesis and its activity was examined as shown in Figure 6.2 and Table 6. As with Fraction C, the results in Figure 6.2 show that VE-cadherin staining was localized at the lateral borders (junctions) of the PDL cells and vWF staining was in diffuse intracellular vesicle-like structures (probably Weibel-Palade bodies) within the PDL cells treated with EBM-2 alone and EBM-2 + TRAP, as also observed previously using HUVEC (Alghisi et al., 2009; Muller et al., 2002). Measurement of the effect of TRAP on PDL cell vasculogenic differentiation showed that, as with Fraction C, the presence of 30 μg/ml of TRAP resulted in 26.2% of the PDL cells exhibiting VE-cadherin staining when cultured in EBM-2 for 5 weeks, compared with only 8.5%-positive cells in the control cultures of EBM-2 alone ($p<0.05$) (Table 6). A similar proportion of VE-cadherin-positive cells were also found in the positive control cultures containing GF (21.7%) ($p<0.05$). Moreover, in the presence of TRAP, 23.1% of the PDL cells were found to also express vWF, a late endothelial protein unique to specific endothelial cell vesicles (Weibel-Palade bodies) (Valentijn et al., 2011), again similar to the proportion of cells in the positive control culture containing GF (17.4%) ($p<0.05$). Thus the TRAP peptide was found to stimulate PDL cell vasculogenic differentiation *in vitro*, comparable with the stimulation observed in the presence of endothelial growth factors.

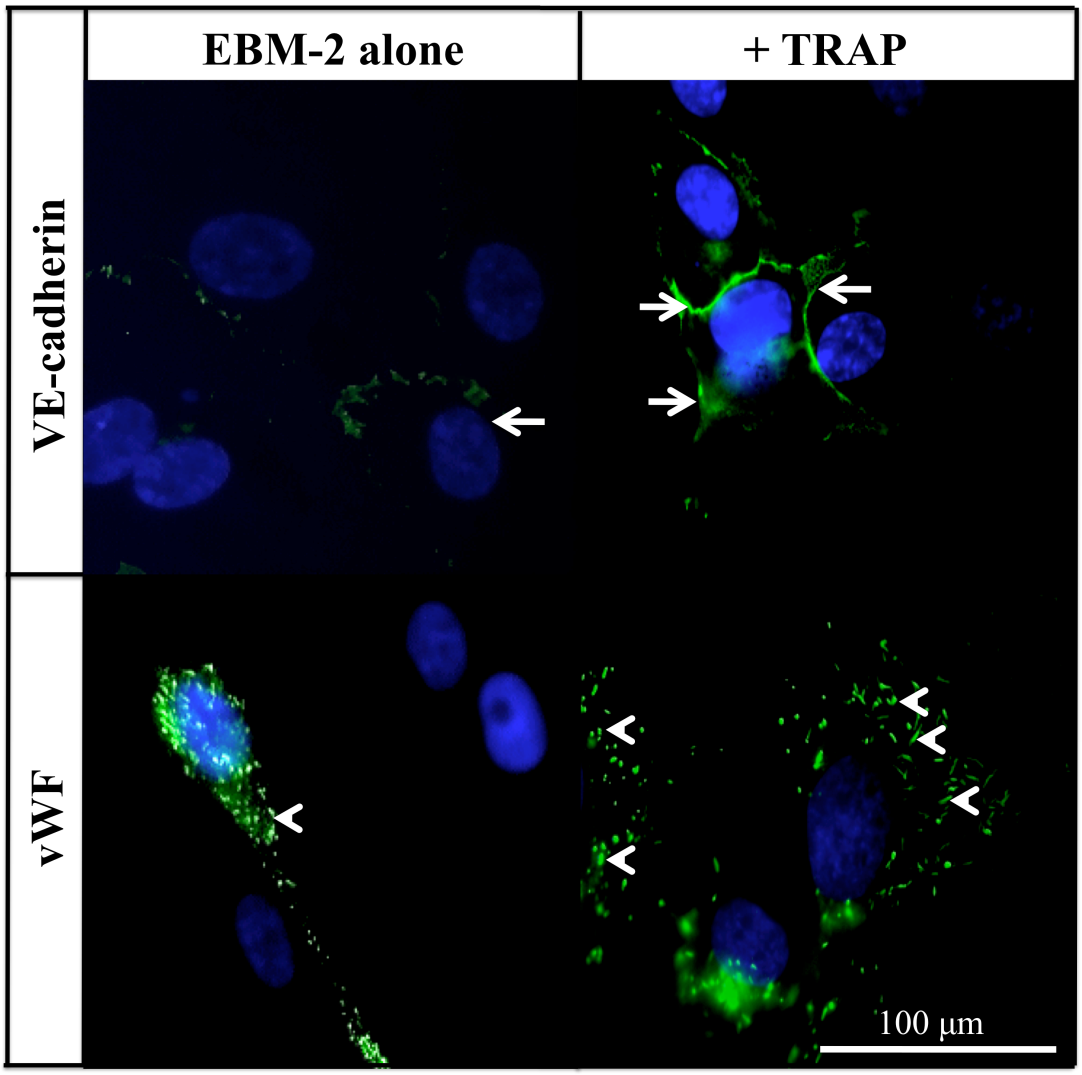


Figure 6.2. Representative micrographs of PDL cells immunostained for VE-cadherin and vWF after culture in EBM-2 alone, EBM-2 + TRAP (30 µg/ml) and EBM-2 + GF for 5 weeks. The nuclei are stained blue with Hoechst dye and the white arrows show the green fluorescence of VE-cadherin staining at the lateral borders (junctions) of the cells. The white arrowheads show the punctate green fluorescence staining of vWF.

Markers	VE-cadherin and vWF-positive cells (% of total cells)		
	EBM-2 alone	**+ TRAP**	**+ GF**
VE-cadherin	8.5±1.3	26.2±3.7*	21.7±3.1*
vWF	7.1±2.8	23.1±5.6*	17.4±4.7*

*Table 6. Effects of TRAP (30 µg/ml) on VE-cadherin and vWF staining of PDL cells cultured for 5 weeks in EBM-2. Cells cultured in EBM-2 + GF was used as a control. The numbers are the % of VE-cadherin- and vWF-positive cells. The values are the means ±SE of three measurements of three separate experiments. *Indicates significant difference compared with EBM-2 alone ($p<0.05$).*

It was shown in Chapter 3 that the expression of endothelial genes was up-regulated when cultured in EBM-2 alone. The present study therefore examined whether addition of TRAP in EBM-2 affects the expression of endothelial genes (VEGFR1, VEGFR2, Tie-1, Tie-2 and VE-cadherin) by PDL cells. The representative RT-PCR gels in Figure 6.3 show that the presence of 30 μg/ml of TRAP increased the mRNA transcript levels of all the endothelial genes examined here, except for VEGFR1. Thus, the presence of TRAP significantly up-regulated the early endothelial gene VEGFR2 (3.9-fold increase) and the late genes Tie-1, Tie-2 (tyrosine kinases essential for angiopoietin-mediated vasculogenesis (Demir et al., 2007)) (6.2- and 6.8-fold increase) and VE-cadherin (2.4-fold increase), compared with cells cultured in EBM-2 alone ($p<0.05$) (Fig. 6.3). Similar results were obtained when the PDL cells were cultured in EBM-2 in the presence of GF (positive control). In contrast, under both conditions the early gene VEGFR1 was unaffected (Fig. 6.3).

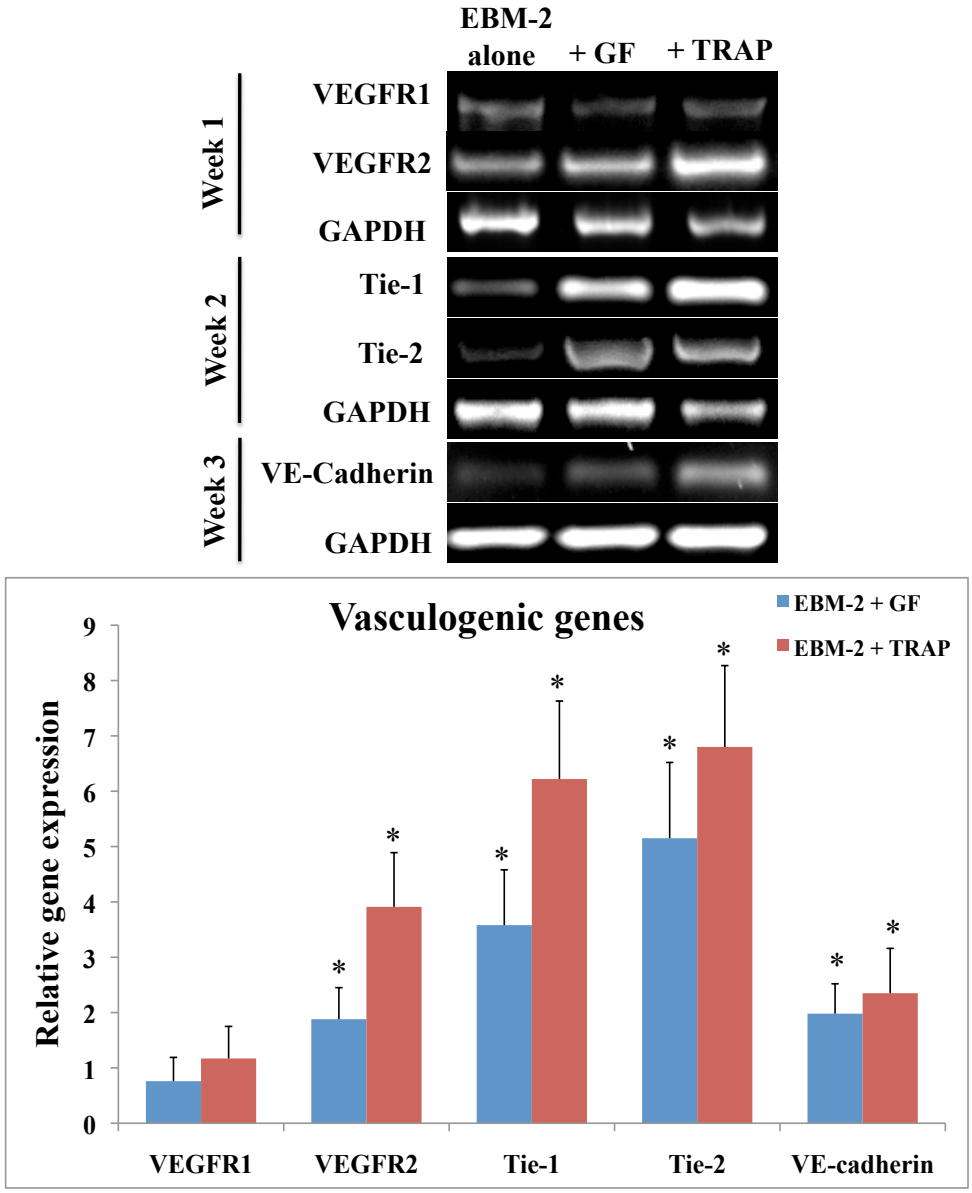


*Figure 6.3. Effect of TRAP on vasculogenic genes of PDL cells. (i) A representative RT-PCR gel showing the expression of the early vasculogenic genes VEGFR1 and VEGFR2 and the late genes Tie-1, Tie-2 and VE-cadherin by PDL cells cultured for 3 weeks in EBM-2 alone, EBM-2 + GF and EBM-2 + TRAP. (ii) The values are the changes in PCR product band intensity relative to GAPDH of cells cultured in the presence of GF and TRAP compared with EBM-2 alone, defined as 1.0. The values are the means ±SE of triplicate measurements. *Indicates significant difference compared with EBM-2 alone ($p<0.05$).*

PDL cells cultured in the presence of 30 μg/ml of TRAP or GF for 5 weeks were also examined for internalization of LDL, a key functional indicator of terminal vasculogenic differentiation (Anderson et al., 1977), by incubating the cells with green fluorescent-tagged acetylated-LDL at 37°C for 2 h and examining the cells under the fluorescence microscope and measuring the relative levels of green fluorescence using FCM. The results in Figure 6.4 (i) show that the PDL cultures incubated in the presence of TRAP and the GF appeared to express a similarly high proportion of fluorescent-labelled cells (an indication of internalized LDL), compared with many fewer positive cells when cultured in GM alone or EBM-2 alone (control cultures). FCM analysis (Figure 6.4 (ii)) showed that approximately 72% of the cells internalized LDL when cultured in EBM-2 + TRAP for 5 weeks and nearly 67% when cultured with GF (positive control). In contrast, only 26.2% of the cells were positive after culture in EMB-2 alone ($p<0.05$; Fig. 6.4 (ii)). These results thus demonstrate that the presence of TRAP resulted in an increase in LDL uptake, similar to that found in the presence of GF.

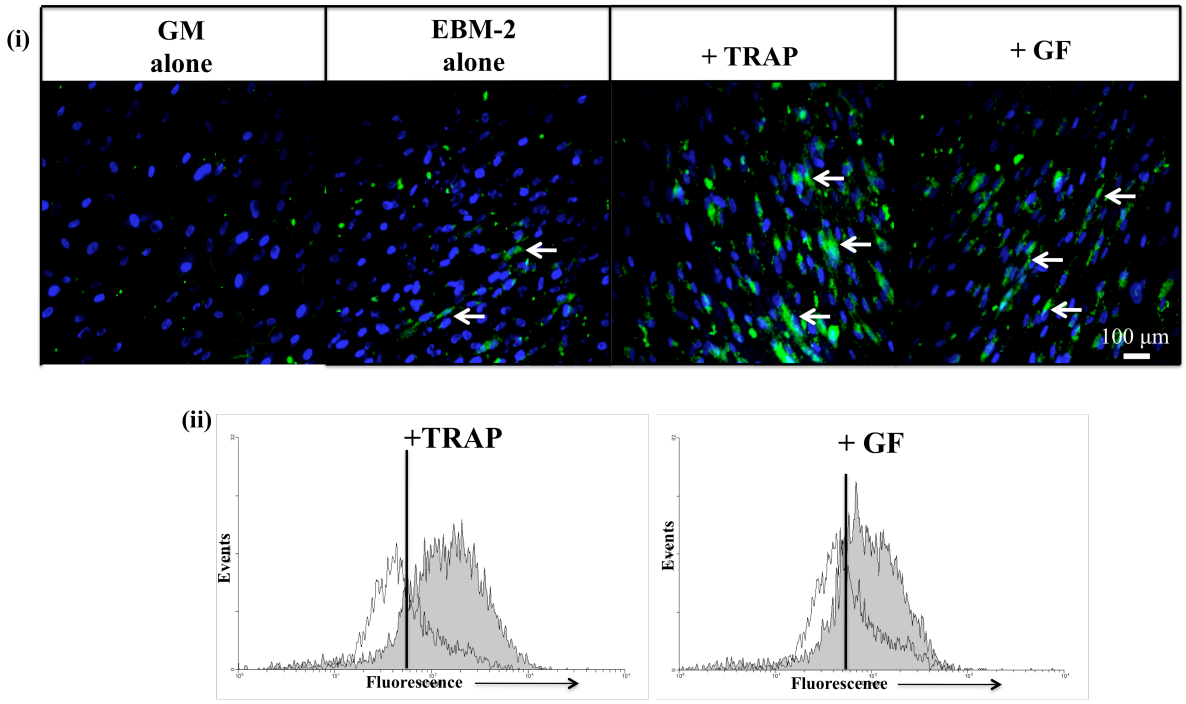


Figure 6.4. Up-take of fluorescent-labelled LDL by PDL cells cultured in EBM-2 alone, EBM-2 + TRAP and EBM-2 + GF for 5 weeks. (i) White arrows show the internalization of LDL as visualized by fluorescence microscopy. (ii) Representative FCM histogram of the levels of intracellular fluorescent-labelled LDL. The shaded profile shows the fluorescence distribution of the TRAP and GF-treated cells, while the open profile shows the control cells cultured in EBM-2 alone. The vertical lines have been selected at a value of 75 fluorescence units. The events on the left side of the vertical lines are considered as non-fluorescent cells (negative cells) and events on the right side of the vertical line are considered as fluorescent cells (positive cells).

6.3.2. Angiogenic differentiation *in vitro*

Once progenitor/stem cells differentiate into endothelial precursors through the process of vasculogenesis, as described above, the process of angiogenesis takes place at the wound site and results in the formation of BV (Brey et al., 2005; Flamme et al., 1997; Nomi et al., 2002). Angiogenic differentiation, the development of an organized network of tubular structures involving the degradation of the basement membrane and the migration of endothelial precursors to the wound site *in vivo*, is a prerequisite for BV formation (Bauer et al., 2005; Yuan et al., 2003). *In vitro* it has previously been shown that such precursors are induced to migrate to wound sites by angiogenic growth factors, including VEGF and EGF, in a 2-dimensional wound healing assay (Staton et al., 2009; Lamalice et al., 2007). In view of previous results that 30 μg/ml of TRAP appears to induce vasculogenic differentiation **(Section 6.3.1)**, the effects of 30 μg/ml of TRAP on PDL cell chemotaxis was examined in a similar 2-dimentional *in vitro* wound healing assay, in which an approximately 1 mm (in width) wound was created by scraping a confluent monolayer culture of PDL cells in a straight line of 1 mm width using a pipette tip. Then, after treatment with TRAP and GF, as described in **Materials and methods**, microscopic images were taken at 0, 6 and 12 h. The results in Table 7 show that the PDL cells incubated with EBM-2 alone (control cultures) migrated over an area of approximately 22500 pixels after 6 h and 32986 pixels after 12 h within the wound site. In contrast, a marked increase in the migration of PDL cells was observed when cultured with EBM-2 + TRAP for 6 h (58360 pixels) and for 12 h (67996 pixels) ($p<0.05$). At this later time complete closure of the wound appeared to have occurred in the presence of TRAP, compared with PDL cells cultured in EBM-2 alone which exhibited only partial closure, as shown in Figure 6.5. Similarly, as with TRAP, a marked increase in PDL cell migration was observed in the positive control of EBM-2 + GF (49053 and 53136 pixels after 6 and 12 h, respectively) ($p<0.05$) (Table 7). Similar results were also observed when HUVEC (positive control cells) were treated with EBM-2 alone, EBM-2 + TRAP and EBM-2 + GF, as shown in Appendix Materials 2.1 and 2.2. These results thus indicate that TRAP induces an initial chemotactic phase comparable with angiogenic GF.

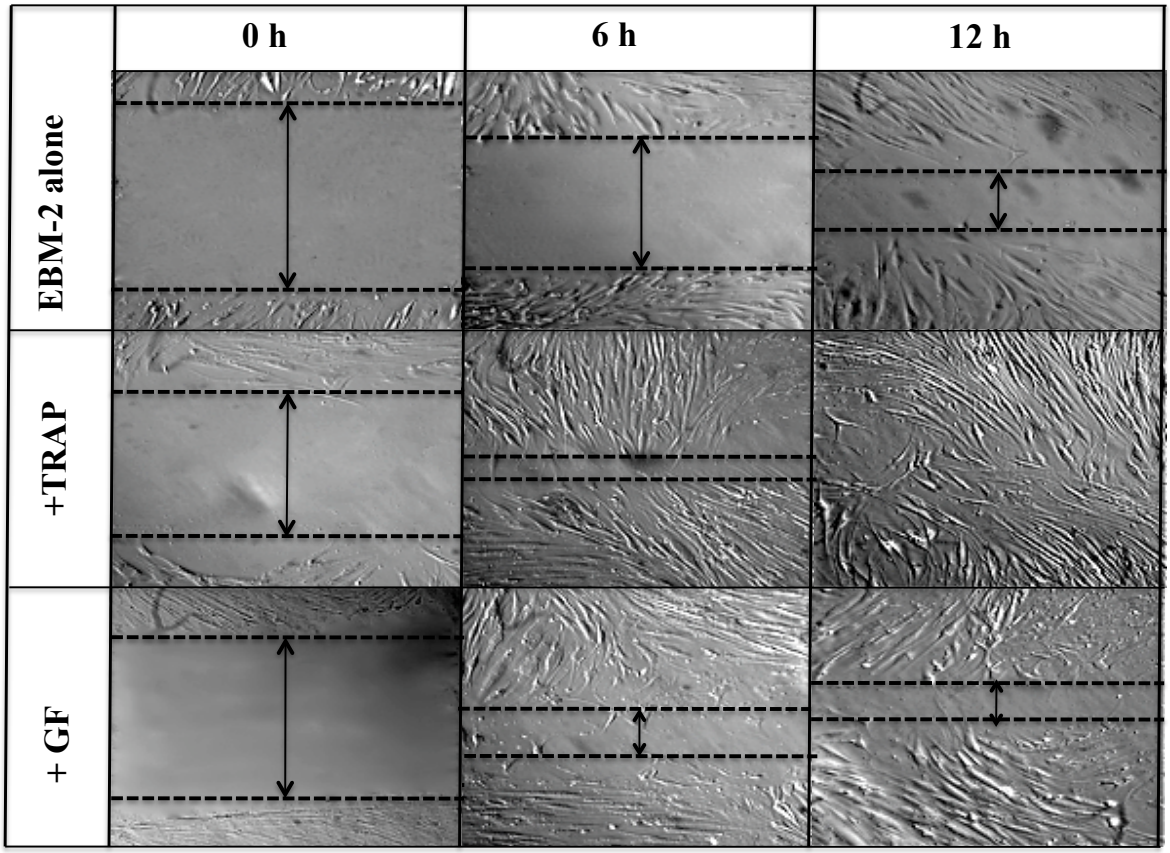


Figure 6.5. Representative microscopic images of PDL cells cultured in EBM-2 alone, EBM-2 + TRAP (30 µg/ml) and EBM-2 + GF for 6 and 12 h followi ng the creation of an in vitro cell wound, as described in ***Materials and methods****. The area between the black dashed lines shows the size of the wound (space without cells) which were measured in pixels. Magnification x 10.*

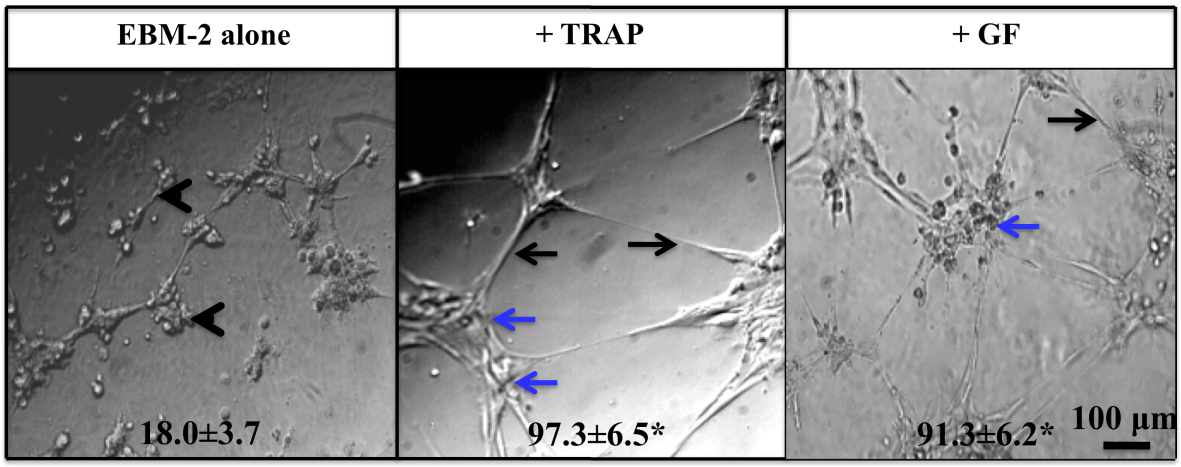
Conditions	Cell migration (in pixels x 10^3)	
	6 h	12 h
EBM-2 alone	22.5±370	33.0±2169
+ TRAP	58.3±1190*	68.0±1479*
+ GF	49.0±2100*	53.1±2244*

Table 7. Effects of TRAP on migration of PDL cells when cultured in EBM-2 alone, EBM-2 + TRAP (30 μg/ml) and EBM-2 + GF for 6 and 12 h after creating the cell wound in vitro. The numbers are the average (of 3 separate experiments) of the measurement of area (in pixel counts x 10^3) of PDL migration (area of wound (no cells) at 0 h minus area of wound at 6 or 12 h in the absence and presence of TRAP and GF).

**Indicates significant difference compared with EBM-2 alone ($p<0.05$).*

In vivo, the accumulation of platelets (thrombocytes) at a fibrin clot provides a growth factor-rich environment that attracts the endothelial precursors to migrate to the wound site and induces their organization into tubular-like structures that ultimately form BV (Chang et al., 2004; Madri et al., 1988). The process of tubule-like structure formation by endothelial precursors has previously been shown morphologically by culturing the cells on a gel of basement membrane proteins with angiogenic factors (Dong et al., 2010; Auerbach et al., 2003; Zhang et al., 2008). The present study therefore examined the effects of TRAP on angiogenic structure formation using an *in vitro* angiogenesis kit in which PDL cells were cultured in EBM-2 on a basement membrane gel layer in a 96-well plate in the absence and presence of TRAP and GF, as described in **Materials and methods**. The results in Figure 6.6 (i) show that a complex polygonal tubular network, morphologically characteristic of angiogenesis, was formed when PDL cells were cultured in EBM-2 in the presence of TRAP for 5 h. In contrast, relatively fewer of these structures were present in control cultures in which PDL cells were cultured in EBM-2 alone (Fig. 6.6 (i)). The branching points of these polygonal tubular-like structures were manually counted and the results demonstrated that in the presence of TRAP there were nearly 97 branch points of the polygonal tubular structures, similar to the 91 branch points observed in the presence of EBM-2 + GF ($p<0.05$; Fig. 6.6 (i)). Cells cultured in EBM-2 alone contained only 18 branch points. Further, PDL cells cultured for an extended period of 15 h in the presence of TRAP demonstrated the formation of distinct elongated BV-like structures (Fig. 6.6 (ii)), in contrast to PDL cells cultured with EBM-2 alone in which these structures were not detected (data not shown). Similar results were also obtained when HUVEC (positive control cells) were cultured on a gel of basement membrane proteins in the presence of EBM-2, EBM-2 + TRAP and EBM-2 + GF, as shown in Appendix Material 2.3. These results thus indicate that TRAP induces PDL cell organization into tubule-like structures in an *in vitro* angiogenesis assay, comparable with the inductive effects of angiogenic GF.

(i)



(ii)

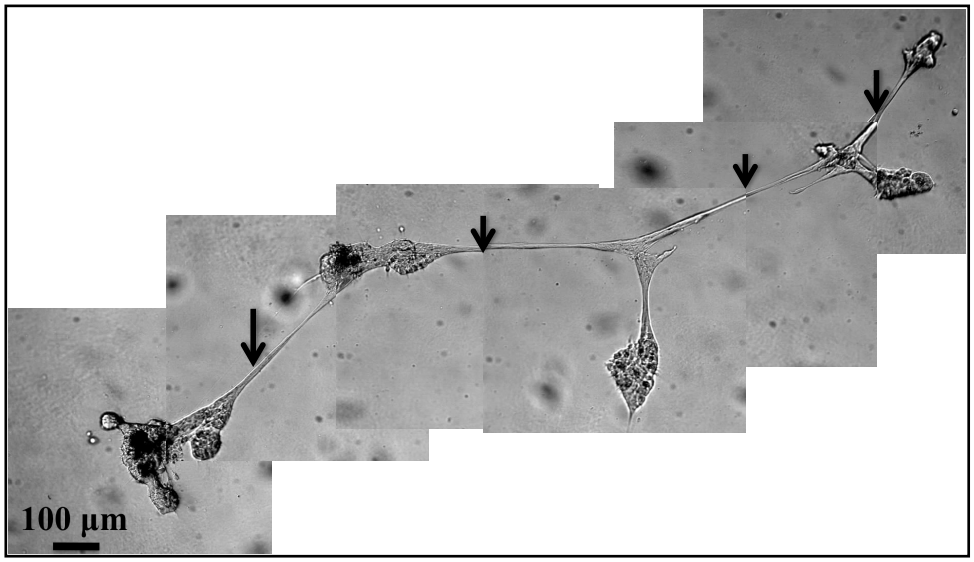


Figure 6.6. Angiogenic structure formation by PDL cells in vitro. (i) PDL cells cultured for 5 h in EBM-2 alone, EBM-2 + TRAP (30 µg/ml) and EBM-2 + GF. The numbers are the angiogenic structure branching points. The black arrows show the polygonal tubule-like structures formed by PDL cells. The blue arrows show branching points of the PDL cells. Black arrowheads show PDL cells that did not form polygonal tubular-like structures. The values are the mean ±SE of five measurements of three separate experiments. (ii) An elongated BV-like structure formed by PDL cells cultured for 15 h in EBM-2 + TRAP. Magnification x 10.

6.3.3. Effects of TRAP on CAM angiogenesis *ex vivo*

The chick embryonic chorio-allantoic membrane (CAM) model has been shown to be a reproducible, efficient and readily accessible for examining the angiogenic potential of drugs and other external factors *ex vivo* (Esch et al., 1985; Rabatti et al., 1995; Auerbuch et al., 2003; Dong et al., 2010). Since TRAP was found to stimulate angiogenic differentiation of mature EC *in vitro*, as shown above, the present study used the CAM model to examine whether TRAP also exhibited angiogenic activity *ex vivo*, as described in **Materials and methods**. The results in Figure 6.7 show that when 7 days old chick embryos were treated with 25 and 50 μg/ml TRAP for 3 days, the filter disks that had been placed over the CAM appeared to be surrounded by capillaries that had sprouted from arteries (Fig. 6.7). Although 1 μg/ml of TRAP had no significant effect on the appearance of such 'allantoic capillaries' (capillaries sprouted from the arteries (AR) of the developing CAM of chick embryo responsible for gas exchange and removal of the waste products; Esch et al., 1985; Rabatti et al., 1995; Auerbuch et al., 2003), compared with the control CAM, the presence of 25 and 50 μg/ml TRAP significantly increased the number of capillaries in the CAM (2.6- and 2.8-fold respectively; $p<0.05$). Increasing the TRAP concentration to 100 μg/ml increased the overall number of capillaries (2.0-fold increase; $p<0.05$) compared with the control CAM, but the tissue near the filter disk did not exhibit extensive sprouted capillaries as observed in the presence of 25 and 50 μg/ml of TRAP, as noted above. These results thus suggest that 25 to 50 μg/ml of TRAP appeared to strongly stimulate BV formation in this *ex vivo* model.

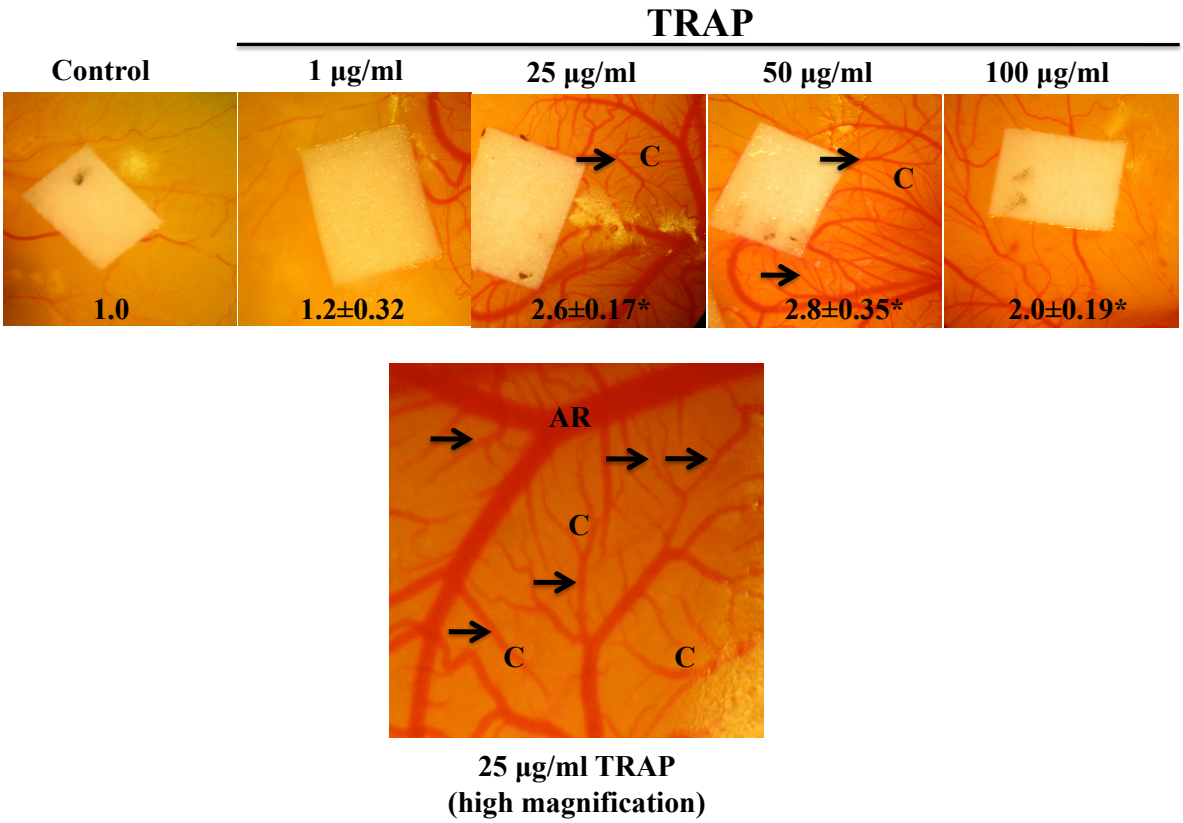


*Figure 6.7. Effects of increasing concentrations of TRAP in angiogenesis ex vivo. BV development after 3 days of treatment with TRAP (1-100 µg/ml) of chick embryo CAM ex vivo. Embryos treated with bovine serum albumin were used as a control. The numbers show the relative capillary density (number of total capillaries in a micrograph) in the presence of TRAP compared with the control CAM. The black arrows show vascular bundle-like 'allantoic capillaries' (C). AR shows an artery from which allantoic capillaries are sprouted. The values are the mean ±SE of measurements of 6 separate experiments. *Indicates significant difference compared with the control (bovine serum albumin), defined as 1.0 ($p<0.05$).*

6.4. Discussion

The process of neovascularization, fundamental in both development and the repair/regeneration of adult tissue (Brey et al., 2005; Flamme et al., 1997; Nomi et al., 2002), involves vasculogenic differentiation of stem cells into endothelial precursors (Brey et al., 2005; Flamme et al., 1997) and angiogenic formation of an organized network of BV-like tubular structures by endothelial precursors (Brey et al., 2005; D'Amore et al., 1987; Flamme et al., 1997). The vasculogenic potential of PDL cells and the effects of EMD on vasculogenic differentiation have previously not been examined. It was shown in Chapter 3 that PDL cells when cultured in media that facilitates vasculogenesis are able to form endothelial-like cells. The present study therefore examined the effects of EMD and recently fractionated EMD components on PDL cells cultured in differentiation media and demonstrated that the low molecular weight Fraction C of EMD strongly stimulated PDL cells to undergo vasculogenic differentiation, whereas the high molecular weight EMD Fraction A (in which Fraction C components are absent) suppressed vasculogenic differentiation. Moreover, the present study also showed for the first time that the main component of Fraction C, a tyrosine-rich 45-amino acid amelogenin peptide (TRAP) derived by N-terminal proteolytic clipping of the full-length amelogenin, appeared to elicit PDL cell vasculogenesis, based on up-regulation of expression of the endothelial markers VEGFR2, Tie-1, Tie-2, VE-cadherin and vWF by the PDL cells and the increased ability of these cells to internalize LDL.

The marked stimulation of vasculogenic differentiation of PDL cells by the TRAP peptide was found to be comparable with the effects of known GF (VEGF and EGF) on vasculogenic differentiation of human umbilical cord blood-derived mesenchymal stem cells and the murine embryonic mesenchymal cell line C3H/10T/1/2, in which the expression of VE-cadherin, vWF, Tie-1 and Tie-2 were also up-regulated (Wang et al., 2010; Gang et al., 2009). In addition, the present study showed that PDL cells cultured in vasculogenic conditions in the presence of TRAP were found to be able to internalize markedly higher levels of acetylated-LDL, indicating that TRAP induced the expression of LDL receptors, a key functional characteristic of EC (Anderson et al., 1977). Notably, the proportions of PDL cells that were able to internalize acetylated-LDL after the incubation with EBM-2 alone and EBM-2 + TRAP were found to be significantly higher than the PDL cell subpopulation that expressed VE-cadherin and vWF after EBM-2 alone and EBM-2 + TRAP treatment. Cholesterol is one of the major building blocks of mammalian cell membrane and notably,

synthesis of cholesterol has been shown to occur to some extent in fibroblasts, in addition to EC, depending on the cellular requirements of the lipids. These cells (EC, fibroblasts) have been shown to express LDL receptors to which LDL ligands bind, internalize and are transported to the lysosomes where the cholesterol esters of LDL are hydrolyzed to make cholesterol freely available for the cells and the receptors are sent back to the plasma membrane (Goldstein et al., 1977). PDL tissue has previously been reported to contain fibroblast-like population, based on elongated spindle shape-like morphological appearance and their ability to secrete substantial amounts of collagen, a major building block of connective tissue (Tencate, 1989; Hassell, 1993; Kuru et al., 1998; Parker et al., 2001), indicating the possibility that some of the LDL receptor-positive cells in the heterogeneous PDL population used here may be of fibroblastic lineage which may at least partly account for the higher proportion of PDL cells being able to internalize LDL, noted above (Anderson et al., 1977; Goldstein et al., 1977).

Under vasculogenic conditions, TRAP was also found to stimulate the expression of the early endothelial marker gene VEGFR2, a tyrosine kinase receptor for the VEGF ligand, the late genes Tie-1 and Tie-2 (tyrosine kinase receptors for the angiopoietins that are expressed exclusively by EC; Jones et al., 2001) and the late gene VE-cadherin, an endothelial cell adhesion molecule, of PDL cells when cultured under selective differentiation-inducing media. In contrast, the early endothelial gene VEGFR1 was found to be unaffected by the TRAP peptide, similar to that previously reported in PDL cells when cultured under non-selective growth conditions in the presence of EMD (Brett et al., 2002). This receptor has been characterized previously as ‘src’ oncogene involved in abnormal cell proliferation and development of a neoplastic cellular phenotype (i.e., high population doubling level, lack of contact inhibition) (Maru et al., 1998; Schwartz et al., 2010). In addition, knockdown of the VEGFR1 gene from human melanoma cells lose the ability of this cell population to undergo unlimited population doubling and to form tumours upon subcutaneous transplantation into mice, suggesting that the elevated levels of VEGFR1 transcripts, which was not found in PDL cells used here, may be of functional importance to the neoplastic cell lines (Frank et al., 2011). Thus, although the reason(s) for lack of VEGFR1 transcript up-regulation in EBM-2 alone and EBM-2 + TRAP-induced PDL cultures are not yet known, but it may partly be due to the non-neoplastic cellular physiology of PDL cells in which the mechanism(s) responsible for the up-regulation of VEGFR1 transcripts might be absent or inactive.

It is now recognized that the process of angiogenesis has two essential steps, endothelial precursor chemotaxis and the organization of endothelial precursors into tubular-like structure (Auerbach et al., 2003; Obeso et al., 1990). The present study therefore investigated the effect of TRAP on PDL cell chemotaxis using the cell monolayer wound healing assay, the results showing that TRAP markedly stimulated the chemotactic activity of PDL cells. Previous studies have also examined the effects of unfractionated (non-heat treated) EMP and heat-treated EMD on chemotaxis/migration of HUVEC and HMVEC *in vitro* (Johnson et al., 2009; Yuan et al., 2003; Bertl et al., 2009; Schlueter et al., 2007). For example, 100 μg/ml of EMP was found to stimulate the highest migration/chemotaxis of human microvascular endothelial cells (HMVEC) *in vitro* (Johnson et al., 2009; Yuan et al., 2003). However, another study observed that 100 μg/ml EMD did not induce HMVEC migration/chemotaxis (Schlueter et al., 2007) and Bertl (2009) reported that 100 μg/ml of EMD also did not stimulate HUVEC migration/chemotaxis *in vitro*. These discrepancies might be partly due to the use of different preparations of EMP and EMD containing different proportions of the constituent proteins, including the TRAP peptide. In addition, some of these studies suggested that the chemotactic effects of EMP are likely to be due to the presence of GF (TGF β and PDGF) in the non-heat treated preparation (Johnson et al., 2009; Yuan et al., 2003), although in the present study the synthetic TRAP peptide was used since Fraction C which was found to stimulate vasculogenic differentiation mainly contains TRAP, and demonstrated that the TRAP peptide in EMD is likely to be at least partly responsible for PDL cell chemotaxis induction. However, it is not yet clear whether the TRAP peptide-induced wound healing observed here was an outcome of cell chemotaxis alone since it may in part also be due to proliferation of these cells. Full healing of *in vitro* PDL cell wound was observed after 12 h in the presence of TRAP, whereas the population doubling time of PDL cells is approximately 18.5 h, suggesting that proliferation of PDL cells is unlikely to be involved in the process of TRAP-induced wound healing.

In Chapter 3 it was shown that the PDL cells when cultured on a basement membrane protein gel could form tubule-like structures with minimal sprouting. Thus, the effects of the TRAP peptide on PDL cell angiogenic structure-formation was examined here, the results showing that TRAP induced PDL cells to form greater numbers of complex polygonal tubule-like structures than found in control cultures (in the absence of TRAP), demonstrating that this synthetic peptide stimulates angiogenesis *in vitro*. In contrast, in a recent study using fractionated components from a non-heat treated preparation of EMP, it was shown that the

high (> 15 kDa) and low molecular (< 6 kDa) weight components of EMP were not responsible for the increase in angiogenic structure formation activity of HMVEC (Johnson et al., 2009). However, the protein fractions used in Johnson (2009) to examine the angiogenic activity were inadequately purified, mainly because the higher molecular weight fraction (> 15 kDa) contained < 6 kDa peptides and the lower molecular weight fraction (< 6 kDa) contained > 15 kDa peptides, compared with the present study which used synthetically prepared peptide.

The angiogenic activity of TRAP was further established using an *ex vivo* chick embryo CAM model, which showed that in the presence of the TRAP peptide an increased number of allantoic vessels were found compared with control eggs treated with bovine serum albumin. Using a murine angiogenesis model, it was shown that both the low (< 6 KDa) and high (<15 kDa) molecular weight fractions of heat-treated EMD exhibited strong angiogenic activity *in vivo*, although the specific active component(s) of these fractions were not identified (Thoma et al., 2011) and both the fractions contained components of differing molecular sizes that may have differential effects on angiogenesis, unlike the synthetic peptide used here.

In conclusion, the present study demonstrates for the first time that the low molecular weight EMD Fraction C promotes vasculogenic differentiation *in vitro* and the components of higher molecular weight Fraction A suppress this process. In addition, the data here shows that the presence of the TRAP peptide is likely to be at least partly responsible for the vasculogenic and angiogenic differentiation *in vitro* and *ex vivo*.

Chapter 7

7.1. Introduction

The PDL, a proprioceptive sensory apparatus of the periodontium, receives dense peripheral sensory nerve innervations from both the trigeminal ganglion and mesencephalic trigeminal nucleus that are responsible for touch, pressure and pain sensations (Nishikawa et al., 2006; Imai et al., 2003). At least two types of sensory nerve endings have been shown to be present in the PDL: (i) nociceptive free nerve endings which respond primarily to injury, sending signals to the brain and spinal cord and enabling localized pain sensation; and (ii) specialized mechanoreceptive endings (ruffini endings), responsible for jaw movement and applied pressure during mastication (Nishikawa et al., 2006; Imai et al., 2003). Two types of glial cells have also been identified in the PDL: (i) astrocytes, responsible primarily for nerve myelination (Nishikawa et al., 2006; Imai et al., 2003) and (ii) specialized glia-associated Schwann cells, responsible for guiding nerve growth during PDL regeneration as well as for nerve myelination (Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997; Bhatheja et al., 2006). The oligodendrocytes, a type of glial cell exclusively found in the central nervous system (CNS), are not present in PDL tissue (Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997; Bhatheja et al., 2006). In the continuously repairing/remodeling environment of the PDL these nerve-associated tissues are of crucial importance, along with connective tissue and BV, for normal healthy periodontal function (Nishikawa et al., 2006; Imai et al., 2003; Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997; Bhatheja et al., 2006).

Damage due to periodontal disease also affects PDL nerves and causes debilitating pain (Imai et al., 2003). A number of growth factors (e.g. glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-4/5) have been used to help repair/regenerate damaged PDL nerves and have achieved some degree of success *in vivo* (Imai et al., 2003, Wakisaka et al., 2003; Harada et al., 2003; Jabbar et al., 2007). However, such growth factors primarily target nerve repair and conversely, not elicit 'full' periodontal regeneration, which also includes PDL, BV, bone and cementum.

Although EMD has been shown to stimulate the osteogenic and chondrogenic differentiation of PDL cells and angiogenic differentiation of HUVEC *in vitro* (Gestrelius et al., 1997; Van der Pauw et al. 2000; Ohyama et al., 2002; Schlueter et al., 2007; Narukawa et

al., 2007a; Narukawa et al., 2007b) and also to promote regeneration of PDL, BV, bone and cementum in animals and humans (Cochran et al., 1999; Donos et al., 2003; Hammarstrom, 1997a; Heijl et al., 1997; Gkranias et al., 2010; Thoma et al., 2011), its effect on PDL nerve cells and nerve tissue are not yet known.

Degree III inter-radicular (furcation) defects in molar teeth in monkeys treated with EMD have been shown to develop functionally oriented cementum with inserting PDL fibres and AB, compared with the untreated defects in which little if any regeneration was observed (Donos et al., 2003). Since this animal model has been of value in investigating the effects of EMD on periodontal regeneration, in the present study it was also used for examining the effect of EMD on PDL nerve regeneration *in vivo*. In addition, since the present study also showed (Chapter 3) that the PDL contains progenitor cells capable, under the appropriate condition *in vitro*, of forming nerve-like cells having a morphology similar to sensory nerve cells (Nishikawa et al., 2006; Imai et al., 2003) and glia-associated astrocytes, oligodendrocytes and Schwann cells, EMD and the EMD Fractions were examined to determine which if any of these components influence the neural and glial differentiation pathways of PDL cells under conditions which facilitate neurogenic and gliogenic differentiation *in vitro*.

7.2. Materials and methods

PDL cell culture and RT-PCR were performed as described in Chapter 2; additional methods used in this chapter are described below.

1.8.1. 7.2.1. Treatment of cells with EMD and the EMD Fractions

EMD, Fraction C and Fraction A were diluted in 0.1% acetic acid and increasing concentrations (1-100 μg/ml) were added to confluent PDL cell cultures, as described below. In addition, the chemically synthesized 45-amino acid TRAP sequence (NH_2-MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPYTSYGYEPMGGW–COOH; provided by Institut Straumann, Basel, Switzerland), a major component of Fraction C, was diluted in water (Thermo Scientific, Basingstoke, UK) and 10 μg/ml added directly to the cells in differentiation medium for each experiment. The concentrations of EMD, EMD Fractions and TRAP were determined from the dose effects on terminal neurogenesis and gliogenesis *in vitro*, as described below.

7.2.2. Effects of EMD and the EMD Fractions on neural differentiation *in vitro*

2 x 10^4 cells were seeded onto poly-L-lysine/laminin coated 8-well chamber slides (Nunc) and cultured in 0.2 ml of GM for 2 days. Neural differentiation medium (NM) (0.2 ml) was then added, consisting of 20 ng/ml EGF, 20 ng/ml bFGF, 4 μM forskolin, 10 μM retinoic acid and 50 ng/ml NT-3 and BDNF in the absence and presence of 1, 3, 10, 30 and 100 μg/ml of EMD, Fraction C and Fraction A. After 5 days the cells were fixed with 4% paraformaldehyde (PFA) (Merck) for 15 min at RT and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% NGS in PBS for 1 h and incubated for 1 h at RT with mouse monoclonal anti-βIII tubulin (1:1000) (R&D Systems, Minneapolis, MN, USA), anti-neurofilament (NF)-L (1:200) (Abcam, Cambridge, UK) (both markers of intermediatory nerve cell filaments expressed at the late stage of nerve cell differentiation; Techawattanawisal et al., 2007; Widera et al., 2007) and anti-protein gene product 9.5 (PGP9.5) (1:100) (Insight Biotechnology Ltd, London, UK), a nerve cell-specific ubiquitin-protein hydrolase expressed at the late stage of nerve cell differentiation (Nandasena et al., 2007), in PBS containing 1% NGS. After incubation with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK),

diluted 1:200 in PBS containing 1% NGS, for 1 h at RT, neuron-like cells were visualized by their green fluorescent staining (for βIII tubulin and NF-L). HRP staining was also carried out for PGP9.5 to examine nerve cell morphology. Immunostaining of the PDL cells for βIII tubulin, NF-L and PGP9.5 was carried out to examine the effects of EMD and the EMD components on terminal stages of neurogenic differentiation of PDL cells. Nuclei were stained blue using Hoechst dye. The proportions of βIII tubulin-, NF-L- and PGP9.5-positive cells were determined by manual counting of 3 separate fields of 3 separate cultures.

Total RNA was extracted from replicate cultures after 2 and 5 days for PCR analysis of the early neurogenic marker NGF and the late markers NF-L and PGP9.5, as described in Chapter 2. Up-regulation of NGF is one of the key early events of neuronal differentiation (Angelastro et al., 2000). In addition to the late neurogenic differentiation events including up-regulation of NF-L and PGP9.5, to determine the effects of EMD components on the early event of neuronal differentiation, the expression of the NGF gene of PDL cells was also examined, as above.

7.2.3. Effects of EMD and the EMD Fractions on glial differentiation

2 x 10^4 cells were seeded onto poly-L-lysine/laminin coated 8-well chamber slides (Nunc) and cultured in GM for 2 days. Glial differentiation medium (GDM) was then added, consisting of 10 ng/ml heregulin β-2 and 4 μM forskolin, in the absence and presence of 1, 3, 10, 30 and 100 μg/ml of EMD, Fraction C and Fraction A. After 7 days the cells were fixed with 4% paraformaldehyde (Merck, Poole, UK) for 15 min at RT and permeabilised using 0.1% Triton X (Sigma) for 15 min at RT. They were then treated with a blocking solution containing 10% NGS in PBS for 1 h and incubated for 1 h at RT with mouse monoclonal anti-glial fibrillary acid protein (GFAP) (1:1000) (R&D Systems, Minneapolis, MN, USA) (an intermediatory filament protein found in the cytoplasm of all three types of glial cells (Hermann et al., 2004; Donato et al., 2007; Chi et al., 2010)) and anti-S-100β (1:100) (Abcam, Cambridge, UK) (a Schwann cell-associated protein found both in the cell nucleus and cytoplasm (Hermann et al., 2004; Donato et al., 2007; Chi et al., 2010)) antibodies in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Invitrogen, Paisley, UK) diluted 1:200 in PBS containing 1% NGS for 1 h at RT, glial-like cells were visualized as green fluorescent (GFAP)-stained astrocytes and red fluorescent (S-100β) stained Schwann-like cells. Immunostaining of the PDL cells for

GFAP and S-100 β was carried out to examine the effects of EMD and the EMD components on terminal gliogenic differentiation of PDL cells. Nuclei were stained blue using Hoechst dye. The proportions of glia-specific GFAP- and S-100β-positive cells were determined by manual counting of 3 separate fields of 3 separate cultures.

Total RNA was extracted from replicate cultures after 2 and 7 days for PCR analysis of the early gliogenic marker Notch1, a key early marker of glial differentiation (Lutolf et al., 2002) and the late markers GFAP and S-100β, as described in Chapter 2. To determine the effects of EMD components on the early events of neuronal differentiation, the expression of the NGF gene by PDL cells was examined, as above.

7.2.4. Statistical analysis

The RT-PCR data are presented as the mean of the triplicate measurements of cells cultured in NM or GDM in the presence of EMD and the EMD Fractions compared with that of cell cultured in NM or GDM alone (defined as 1.0). One-way ANOVA followed by post-hoc Bonferroni test set for $p<0.05$ (for multiple comparison) was used for statistical analysis (SPSS 12.0 software, Chicago, IL).

7.3. Results

7.3.1. Effects of increasing concentrations of EMD and the EMD Fractions on PDL cell neurogenesis *in vitro*

It was shown in Chapter 3 that PDL cells cultured in NM alone stained positive for the neuronal intermediatory filament marker βIII tubulin and exhibited nerve cell-like morphology *in vitro*. To determine possible effects of EMD and the EMD Fractions the present study cultured PDL cells in NM in the absence and presence of EMD, Fraction C and Fraction A for 5 days and immunostained for βIII tubulin, as described in **Section 7.2**, to examine whether these protein preparations affect the expression of this antigen. The representative micrographs in Figure 7.1 shows that PDL cells cultured in the presence of NM alone and NM + Fraction C exhibited βIII tubulin-positive nerve cell-like morphology, elongated axon with dendritic projections, as shown previously (Kriegstein et al., 1983; Techawattanawisal et al., 2007), in contrast to cells cultured with NM + EMD and NM + Fraction A which did not express βIII tubulin or exhibit nerve cell-like morphology. To examine the effects of increasing concentrations of EMD, Fraction C and Fraction A PDL cells cultured for 5 days in NM in the absence and presence of 1-100 μg/ml of EMD and the EMD Fractions. PDL cells cultured in NM alone were found to contain 7.1% of βIII tubulin-positive nerve-like cells whereas cultures in non-differentiation-inducing GM alone contained no positive cells (Chapter 3). The results in Table 8 show that in the presence of EMD (at all concentrations) there was a very low proportion of PDL cells which expressed βIII tubulin (0.0 to 1.7%), significantly lower compared with cells cultured in NM alone (7.1% positive cells) ($p<0.05$). In addition, all concentrations of Fraction A also appeared to suppress the proportion of βIII tubulin-positive cells (0.0 to 1.1% positive cells; Table 8), significantly lower compared with cells cultured in NM alone (7.1% positive cells) ($p<0.05$). In marked contrast to this apparently inhibitory effect of EMD and Fraction A, the results in Table 8 show that increase in the concentration of Fraction C from 1 to 3 μg/ml increased the number of βIII tubulin-positive cells from the control levels (7.9% to 11.6%), while at 10 μg/ml of Fraction C the proportion of positive cells increased further, to 16.9%. However, when the concentration of Fraction C was increased to 30 and 100 μg/ml the proportion of βIII tubulin-expressing cells did not increase further and remained at the same high level (15.4 and 16.2% positive cells, respectively). These results thus indicate that Fraction A suppressed PDL cell neural differentiation and this sub-fraction was not examined further. However, Fraction C

was found to actively promote neural differentiation and further experiments were therefore carried out to clarify the activity of this Fraction by using the TRAP peptide, the main component of Fraction C, which was obtained by chemical synthesis.

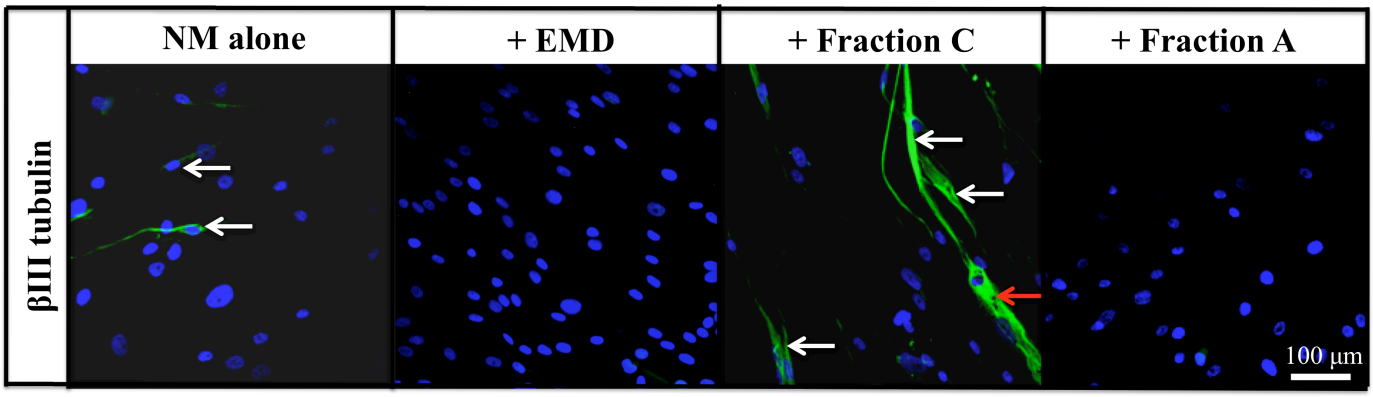


Figure 7.1. Representative micrographs of PDL cells immunostained for βIII tubulin after 5 days of culture in NM alone, NM + EMD (100 µg/ml), NM + Fraction C (10 µg/ml) and NM + Fraction A (100 µg/ml). The nuclei are stained blue with Hoechst dye and the white arrows show βIII tubulin-positive dendritic projections. Red arrow shows βIII tubulin-positive elongated axon of a nerve-like cell. Magnification x 20.

Conc. (µg/ml)	βIII tubulin-positive cells (% of total cells[§])		
	EMD	**Fraction C**	**Fraction A**
1	1.7±1.0*	7.9±2.5	1.1±1.0*
3	1.6±0.7*	11.6±2.0*	0.9±0.4*
10	1.3±0.8*	16.9±1.7*	0.0
30	0.8±0.4	15.4±3.4*	0.0
100	0.0	16.2±2.4*	0.0

Table 8. Effects of increasing concentrations of EMD and the EMD Fractions on βIII tubulin expression by PDL cells. PDL cells were cultured for 5 days in NM in the presence of 1-100 µg/ml of EMD, Fraction C and Fraction A and stained for βIII tubulin, as described in the ***Materials and methods****. The numbers are the % of βIII tubulin-positive cells. The values are the means ±SE of three measurements of three separate experiments. *Indicates significant difference compared with NM alone ($p<0.05$).*

[§]Control cultures in NM alone had 7.1±1.9% βIII tubulin-positive cells

7.3.2. Effects of TRAP on neural differentiation of PDL cells

As demonstrated above **(Section 7.3.1)**, 10 μg/ml of Fraction C markedly increased the apparent neurogenic differentiation of the PDL cells *in vitro*. Since the TRAP peptide has previously been reported to be the main component of Fraction C (Mumulidu et al., 2007), the neurogenic activity of a synthetic 45-amino acid TRAP peptide was examined by culturing PDL cells in NM in the absence and presence of 10 μg/ml of TRAP and immunostained for nerve intermediatory filament markers βIII tubulin and NF-L and nerve cell-specific ubiquitin hydrolase protein PGP9.5. The representative immunomicrographs in Figure 7.2 and quantitative analysis of immunostained cells in Table 9 indicate that in the presence of 10 μg/ml of TRAP approximately 20% of the PDL cells were found to be positive for βIII tubulin, NF-L and PGP9.5 when cultured in NM for 5 days, significantly higher than the cells cultured in NM alone (7.1, 3.2 and 2.8% positive cells, respectively) ($p<0.05$).

Conventional RT-PCR was carried out for NGF (neuronal marker pivotal for the early events of neural differentiation; Angelastro et al., 2000), NF-L and PGP9.5 to examine whether addition of TRAP in NM affects these early and late neuronal genes. The representative RT-PCR gel in Figure 7.3 shows that the presence of 10 μg/ml of TRAP elevated the mRNA transcripts of the early and the late neuronal genes examined here. Thus, results in Figure 7.3 show that, in the presence of TRAP, PDL cells expressed significantly elevated levels of the early neuronal gene NGF (5.3-fold higher than GM alone), compared with cells cultured in NM alone (2.2-fold higher than GM alone) ($p<0.05$). Similarly, the TRAP peptide markedly stimulated the late gene NF-L (7.0-fold increase), compared with cells cultured in NM alone (4.2-fold increase) ($p<0.05$). The PDL cultures did not express transcripts of the late neuronal gene PGP9.5 under growth conditions (in GM alone), although, cells cultured in NM in the presence of TRAP expressed an elevated level of this gene (4.8-fold higher than NM alone) ($p<0.05$). These results thus indicate that TRAP stimulates both early and late neuronal genes and that the presence of synthetic TRAP, similar to Fraction C, promoted differentiation of PDL cells to the nerve-like cells.

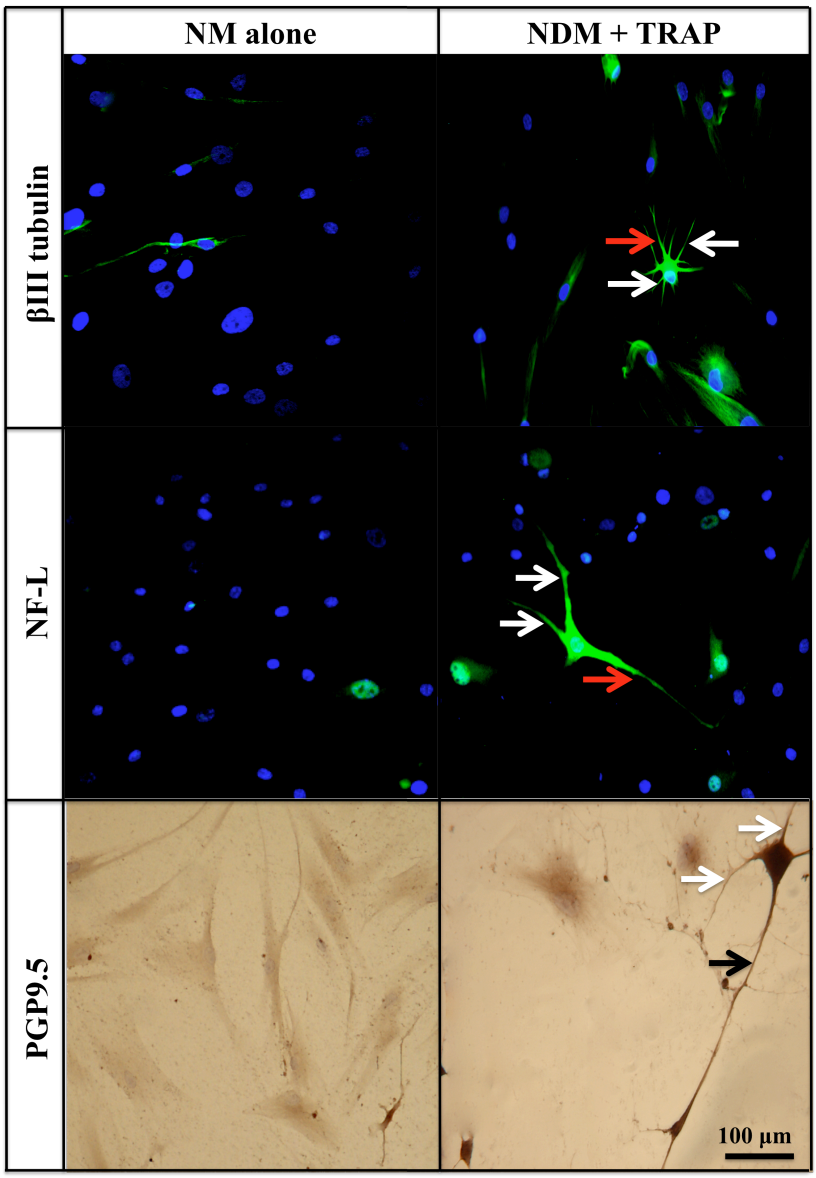


Figure 7.2. Representative micrographs of PDL cells immunostained for βIII tubulin, NF-L and PGP9.5 after culture for 5 days in NM in the presence of TRAP (10 µg/ml). βIII tubulin and NF-L-positive cells are visualized as green fluorescent cells (Magnification x 20). PGP9.5-positive cells are visualized as HRP stained cells (Magnification x40). HRP staining was used for the PGP-9.5 to visualize clear morphological appearance of the cells. Nuclei of the cells (stained for βIII tubulin and NF-L) are stained blue with Hoechst dye. The red arrows show elongated axons of the βIII tubulin, NF-L and PGP9.5-positive cells. The white arrows show multiple dendritic projects.

Markers	βIII tubulin, NF-L and PGP9.5-positive cells (% of total cells)	
	NM alone	NM + TRAP
βIII tubulin	7.1±1.9	20.4±2.3*
NF-L	3.2±0.9	19.3±2.5*
PGP9.5	2.8±1.0	19.1±1.9*

*Table 9. Effects of TRAP on βIII tubulin, NF-L and PGP9.5 staining of PDL cells cultured for 5 days in NM in the presence of 10 μg/ml of TRAP, as described in the **Materials and methods**. The numbers are the % of βIII tubulin, NF-L and PGP9.5-positive cells. The values are the means ±SE of three measurements of three separate experiments. *Indicates significant difference compared with NM alone ($p<0.05$).*

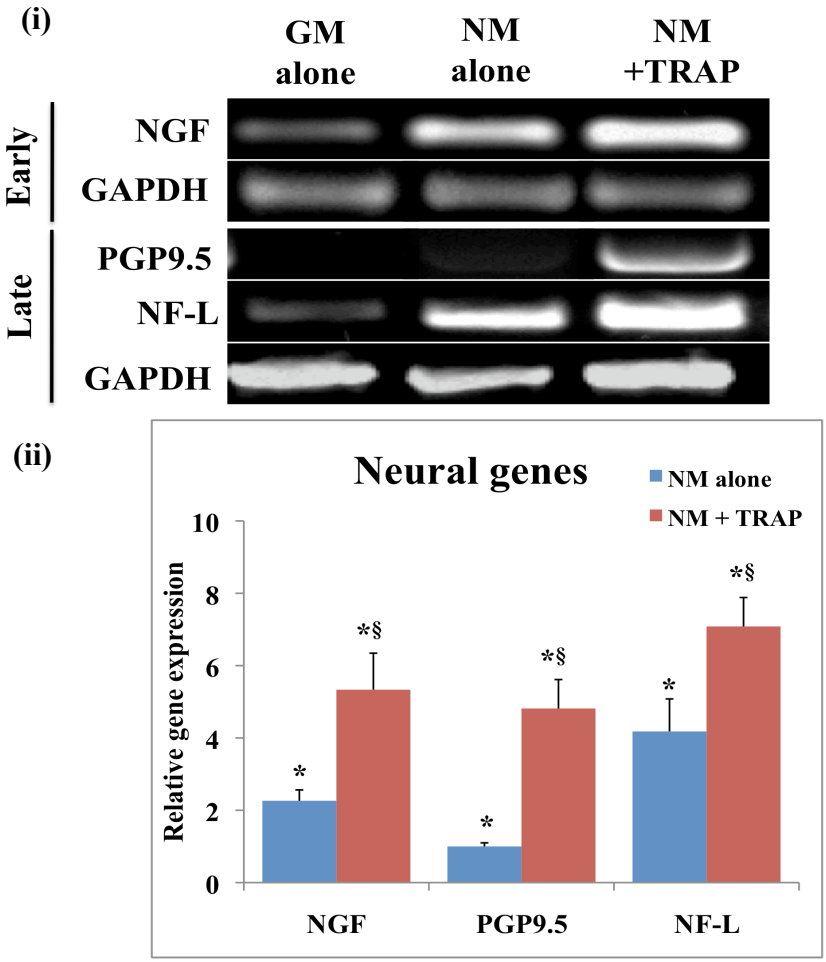


*Figure 7.3. Effect of TRAP on neuronal genes of PDL cells. (i) A representative RT-PCR gel showing the expression of the early neural gene NGF and the late genes PGP9.5 and NF-L by PDL cells cultured for 2 and 5 days, respectively, in GM alone, NM alone and in the presence of NM + TRAP. (ii) The values are the changes in PCR product band intensities relative to GAPDH obtained of cells cultured in NM alone and NM with TRAP compared with GM alone, defined as 1.0. PDL cells cultured in GM alone did not express PGP9.5, thus for this particular gene the values obtained from cells cultured in NM alone were defined as 1.0. The values are the means ±SE of triplicate measurements. * Indicates significant difference compared with GM alone ($p<0.05$); § Indicates significant difference compared with NM alone.*

7.3.3. Effects of increasing concentrations of EMD and the EMD Fractions on PDL cell gliogenesis *in vitro*

As noted above, glial cells are essential for myelinating nerve cells and enabling them to communicate via electric (action potential) and chemical (neurotransmitter) signals (Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997; Bhatheja et al., 2006). They have also been reported to be involved in the process of nerve regeneration by secreting essential neuronal growth factors such as NGF and GDNF (Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997; Bhatheja et al., 2006). In the present study the cell population obtained from PDL tissue was found to have the potential to differentiate into peripheral nervous system (PNS)-associated glial cells (astrocytes and Schwann cells) as well as CNS-associated glial cells (oligodendrocytes) *in vitro* (Chapter 3) when cultured under gliogenic conditions for 10 days, although CNS-associated oligodendrocytes have not been identified histologically in PDL tissue (Atsumi et al., 1999b; Hiroshima et al., 1998; Youn et al., 1997; Bhatheja et al., 2006), indicating that the PDL may have the gliogenic precursors capable of forming all three glial cell types. The results in **Sections 7.3.2 and 7.3.3** indicated that Fraction C and the TRAP peptide have the ability to induce neural differentiation of PDL cells *in vitro,* although their effects on gliogenic differentiation of PDL cells have not previously been examined. The present study therefore examined whether EMD component(s) affect PDL cell gliogenesis *in vitro* when the cells were cultured in GDM in the absence and presence of EMD, Fraction C and Fraction A and, after 7 days, immunostained for GFAP, an intermediate filament protein found in all three types of glial cells (Hermann et al., 2004; Donato et al., 2007; Cora et al., 2009). The representative immunomicrographs in Figure 7.4 indicate that PDL cells cultured in GDM alone and in GDM + Fraction C exhibited a morphology similar to astrocytes, cells with 3-10 dendritic projections, and also a Schwann cell-like population, cells with two dendritic projections at opposite ends of the soma, which were also found to be positive for the glial marker GFAP. In contrast, PDL cells cultured in GDM in the presence of EMD and Fraction A did not exhibit this morphology or GFAP staining (Fig. 7.4). Notably, none of these cultures (GDM alone and GDM + EMD and the EMD Fractions) contained cells which were morphologically similar to oligodendrocytes (glial cells consisting of more than 20-30 dendritic projections) after 7 days (Hermann et al., 2004; Donato et al., 2007; Chi et al., 2010).

The effects of increasing concentrations (1-100 μg/ml) of EMD, Fraction C and Fraction A on PDL cell gliogenesis were examined by culturing PDL cells in GDM in the absence and presence of 1, 3, 10, 30 and 100 μg/ml of EMD and the EMD Fractions for 7 days, then immunostaining for GFAP. Measurement of the proportion of GFAP-positive cells shows that PDL cells cultured for 7 days in GDM alone produced 15.8±2.4% of GFAP-positive glial-like cells (Table 10), whereas cells cultured in non-differentiation-inducing GM did not contain any GFAP-positive cells. In the presence of all concentrations of EMD (1-100 μg/ml) and Fraction A (1-100 μg/ml) very few GFAP-positive glial-like cells were observed (1.2% to 4.1%), as shown in Table 10, significantly lower compared with GDM alone (15.8%; $p<0.05$). In marked contrast, even the lowest concentration of Fraction C (1 μg/ml) markedly increased the proportion of GFAP-positive cells in the culture, to 17.2%, while 10 μg/ml of Fraction C produced the highest number of positive cells (28.3%) (Table 10). Further increasing the concentration of Fraction C to 30 and 100 μg/ml did not result in any further increase in the high proportion of GFAP-positive cells in the cultures. These results thus show that Fraction C significantly enhances the proportion of cells expressing the glial cell marker GFAP by PDL cells *in vitro*, and further experiments were therefore carried out to clarify the activity of this Fraction by using the TRAP peptide. These results also indicate that Fraction A suppresses PDL cell neural differentiation and this sub-fraction was therefore not examined further.

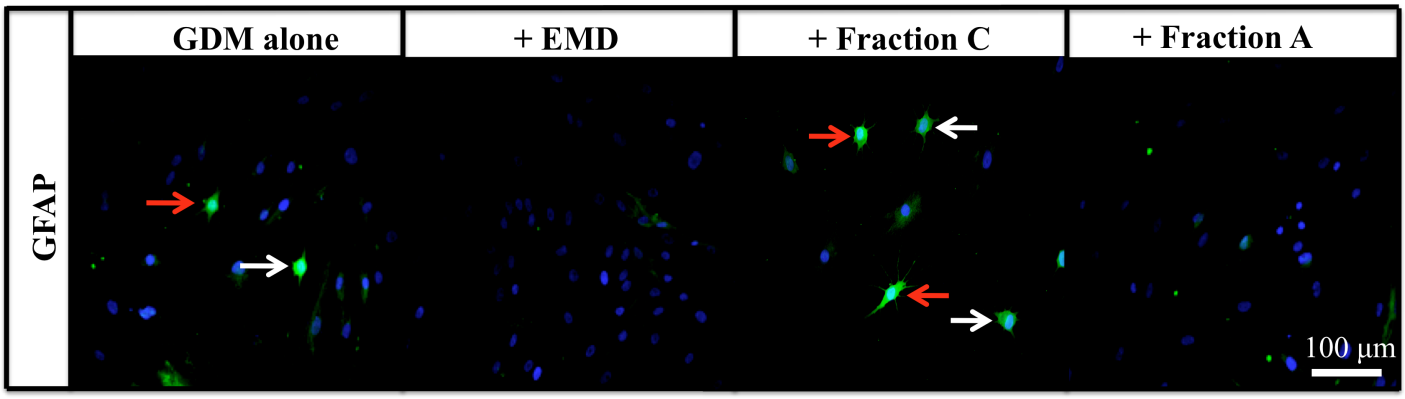


Figure 7.4. Representative micrographs of PDL cells immunostained for GFAP after culture in GDM alone, GDM + EMD (100 µg/ml), GDM + Fraction C (10 µg/ml) and GDM + Fraction A (100 µg/ml) for 7 days. The nuclei are stained blue with Hoechst dye. The white arrows show GFAP-positive cells with Schwann cell-like morphology. The red arrows show GFAP-positive cells with astrocyte-like morphology. Magnification x 20.

Conc. (µg/ml)	GFAP-positive cells (% of total cells)		
	EMD	**Fraction C**	**Fraction A**
1	3.4±2.1*	17.2±2.6	4.1±2.0*
3	2.5±3.8*	19.1±3.8	4.0±3.4*
10	1.2±0.5*	28.3±4.8*	1.5±2.1*
30	0.0	27.1±3.5*	0.0
100	0.0	27.8±2.8*	0.0

*Table 10. Effects of increasing concentrations of EMD and the EMD Fractions on GFAP expression of PDL cells. GFAP staining of PDL cells cultured for 7 days in GDM in the presence of 1-100 µg/ml of EMD, Fraction C and Fraction A, as described in the **Materials and methods**. The numbers are the % of GFAP-positive cells. The values are the means ±SE of three measurements of three separate experiments. *Indicates significant difference compared with GDM alone ($p<0.05$).*

[§]Control culture in GDM alone had 15.8±2.4% GFAP-positive cells

7.3.4. Effects of TRAP on glial differentiation of PDL cells

It was demonstrated in **Section 7.3.4)** that 10 μg/ml of Fraction C markedly increased the apparent gliogenic differentiation of the PDL cells *in vitro*. Moreover, the cells expressing the glial cell marker GFAP in the presence of Fraction C exhibited a morphology similar to astrocytes and Schwann cells. The present study therefore examined the effects of the TRAP peptide, a main component of Fraction C (Mumulidu et al., 2007), on the gliogenic activity of PDL cells *in vitro*, by culturing the cells in GDM in the absence and presence of 10 μg/ml of synthetic TRAP for 7 days followed by immunostaining for GFAP, an intermediate filament marker present in all three types of glial cells, and S-100β, a Schwann cell-specific marker. The results in Figure 7.5 indicate that, in the presence of 10 μg/ml of TRAP GFAP-positive cells were present which exhibited an astrocyte (glial cells with 3-10 dendritic projections) and a Schwann cell-like morphology (two dendritic projections at opposite end of the soma), whereas, S-100β-positive cells exhibited a morphological appearance similar to Schwann cells, as described previously (Chi et al., 2010). It is notable that the cells with morphology similar to oligodendrocytes, glial cells containing more than 20-30 dendritic projections, were not found in the control cultures (GDM alone) or in TRAP peptide-induced cultures (Fig. 7.5). Measurement of GFAP-positive cells showed that 26.3% of the cell population were positive for this marker when cultured in GDM + TRAP for 7 days, significantly higher than PDL cells cultured in GDM alone (15.8%) ($p<0.05$). Furthermore, nearly 21% of the PDL cells cultured in the presence of TRAP were found to also be positive for the Schwann cell marker S-100β, compared with the cells cultured in GDM alone (13.4%) ($p<0.05$) (Table 11).

Conventional RT-PCR was carried out for Notch1 (a glial cell protein pivotal for the early events of glial differentiation; Angelastro et al., 2000), and the late genes GFAP and S-100β to examine whether addition of TRAP in GDM affects the early and the late glial gene regulation of PDL cells. The representative RT-PCR gel in Figure 7.6 shows that the presence of 10 μg/ml of TRAP elevated the mRNA transcripts of the early and the late glial genes examined here. Thus, in the presence of TRAP, PDL cells expressed significantly elevated levels of the early glial gene Notch1 (3.2-fold higher than GM alone), compared with cells cultured in GDM alone (1.9-fold higher than GM alone) ($p<0.05$). Similarly, the TRAP peptide markedly stimulated the late glial gene GFAP (3.8-fold increase), compared with cells cultured in GDM alone (2.5-fold increase) ($p<0.05$). PDL cells cultured in the presence of

TRAP expressed significantly higher levels of the Schwann cell-associated gene S-100β (2.7-fold increase), compared with the cells cultured in GDM alone (1.6-fold increase) (Fig. 7.6). These results thus indicate that TRAP stimulates both the early and the late glial genes and that the presence of synthetic TRAP, similar to that of Fraction C, promoted differentiation of PDL cells to astrocyte and Schwann-like cells.

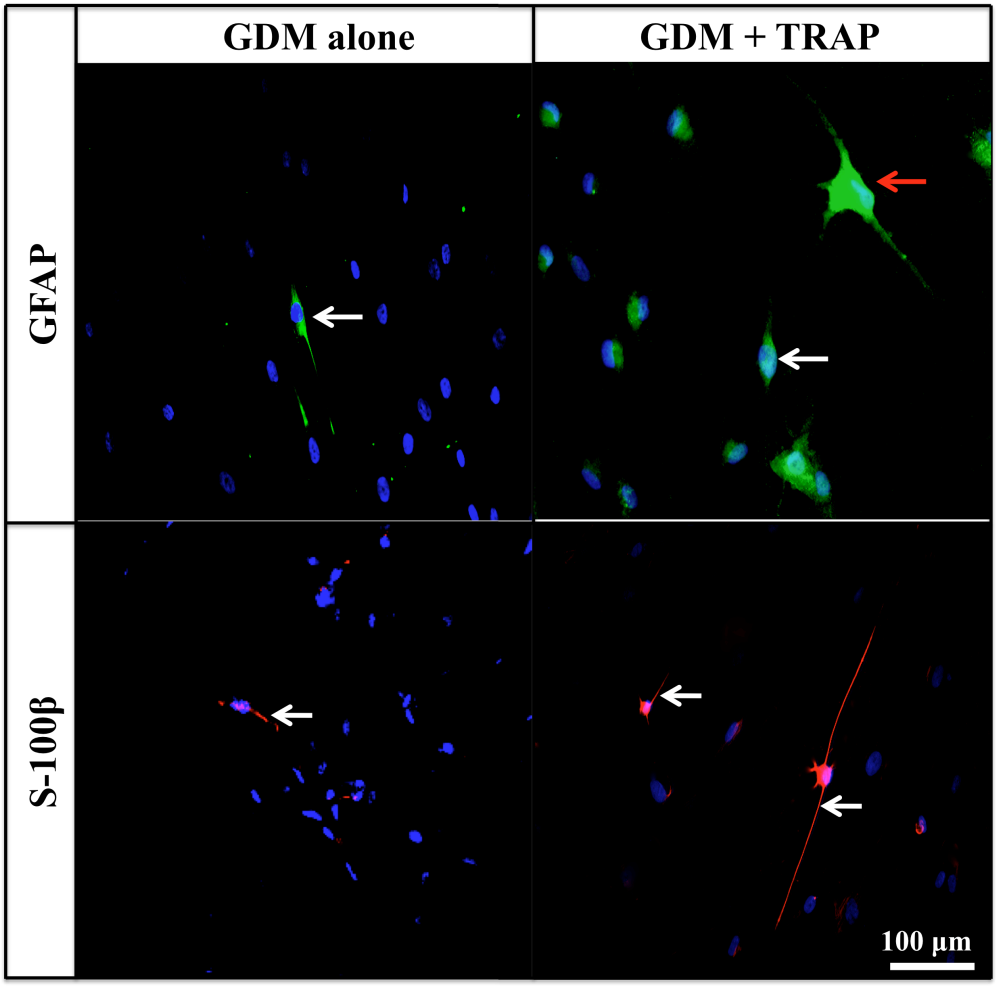


Figure 7.5. Representative micrographs of PDL cells immunostained for GFAP and S-100β after culture in GDM in the presence of TRAP (10 µg/ml) for 7 days. Nuclei of the cells are stained blue with Hoechst dye. GFAP-positive cells are visualized as green fluorescent cells and S-100β-positive cells are visualized as red fluorescent cells. The white arrows show cells with Schwann cell-like morphology (both GFAP and S-100β-positive cells) and the red arrows show GFAP-positive cells with astrocyte-like morphology. Magnification x20.

Markers	GFAP and S-100β-positive cells (% of total cells)	
	GDM alone	GDM + TRAP
GFAP	15.8±2.4	26.3±3.6*
S-100β	13.4±1.1	21.3±2.4*

*Table 11. Effects of TRAP on GFAP and S-100β expression of PDL cells cultured for 7 days in GDM in the presence of 10 μg/ml of TRAP, as described in the **Materials and methods**. The numbers are the % of GFAP and S-100β-positive cells. The values are the means ±SE of three measurements of three separate experiments. *Indicates significant difference compared with GDM alone ($p<0.05$).*

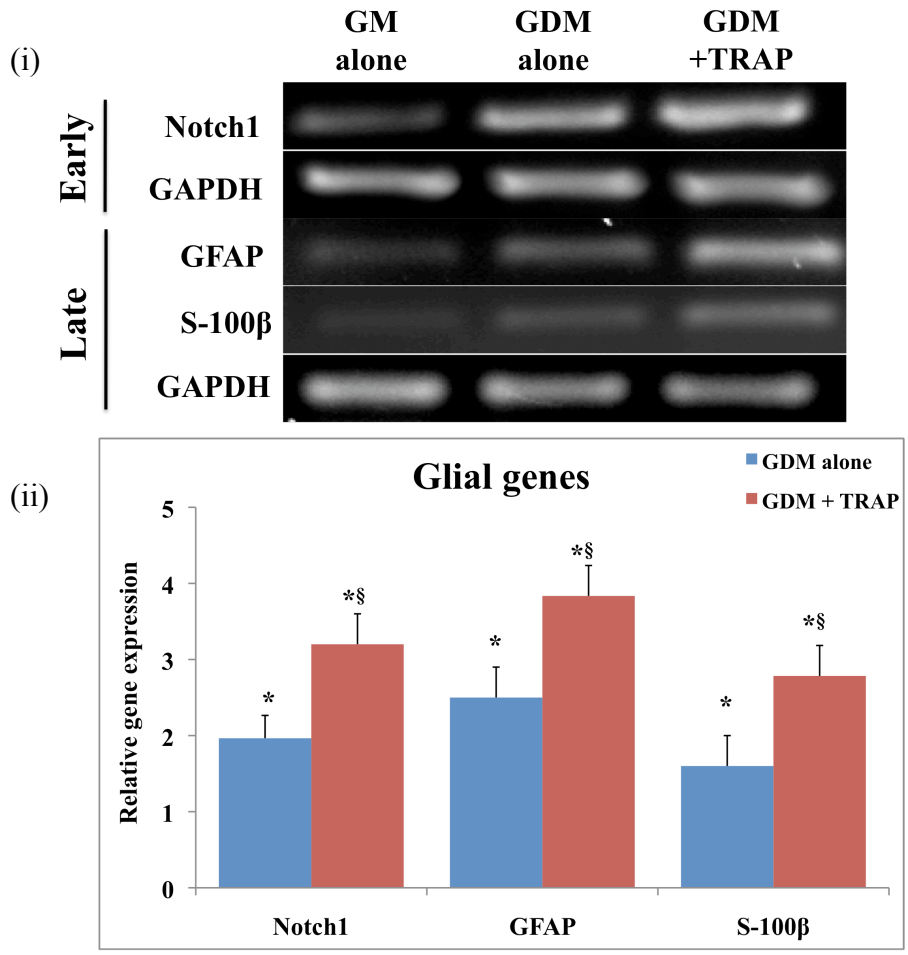


*Figure 7.6. Effect of TRAP on glial gene expression by PDL cells. (i) A representative RT-PCR gel showing the expression of the early gliogenic gene Notch1 and the late genes GFAP and S-100β by PDL cells cultured for 2 and 7 days, respectively, in GDM in the presence of TRAP. (ii) The values are the changes in PCR product band intensity relative to GAPDH obtained in GDM and GDM with TRAP compared with GM alone, defined as 1.0. The values are the means ±SE of triplicate measurements. * Indicates statistically significant difference compared with GM alone ($p<0.05$); §Indicates statistically significant difference compared with GDM alone.*

7.4. Discussion

The PDL contains dense sensory nerves that are responsible for the rapid and efficient conduction of nerve impulses across the axonal bundles, the sensory signals are then transmitted to the brain (Nave et al., 2008). Unlike dental pulp nerves that react to pain only, the periodontal axonal bundles have been shown to contain receptive sites for touch and pressure sensations, orthodontic movements/tooth positioning and jaw movements as well as pain (Matthews et al., 1975; Taylor, 1990). Tissue damage resulting from periodontal disease also affects PDL sensory nerves and causes debilitating pain (Imai et al., 2003). Several attempts have therefore been made to elicit PDL nerve repair, including by the use of nerve cross-anastomosis (a surgical procedure in which damaged periodontal nerve connections are re-sutured with non-specific peripheral nerves) and by the use of growth factors (e.g. GDNF, BDNF, Neurotrophin-4/5) (Imai et al., 2003, Wakisaka et al., 2003; Harada et al., 2003; Jabbar et al., 2007). Although some degree of success has been obtained, these therapies were focused primarily on the nerve regeneration aspect of periodontal therapy. In contrast, although EMD has been extensively studied for its effects on periodontal tissue regeneration involving PDL, BV, bone and cementum (Donos et al., 2003; Sculean et al., 2004; Sculean et al., 2006; Gkranias et al., 2010), its ability to regenerate periodontal nerves is not yet clear. The present study therefore examined whether EMD and the EMD components promoted PDL cell nerve differentiation *in vitro*.

The results in Chapter 3 demonstrated that βIII tubulin-positive cells with the morphological appearance of bipolar sensory nerve-like cells were present when PDL cells were cultured under neurogenic conditions and similarly, under gliogenic conditions, GFAP-, O4- and S-100β-positive cells were found which exhibited a glial-like cell (astrocytes, oligodendrocytes, Schwann cells) morphology. However, as found here, the addition of EMD and the higher molecular weight Fraction A completely ablated both the neural and glial differentiation of the PDL cells *in vitro.* In contrast, both the naturally occurring (Fraction C) and the TRAP isoform strongly stimulated neurogenesis of PDL cells, with nerve-like cells in these TRAP-induced cultures exhibiting a sensory neuron-like morphology and suggesting that this 5.3 kDa isoform is likely to be having neurogenic activity. Amelogenin and amelogenin isoforms, the main components of EMD, are known to be secreted during tooth formation (ten Cate et al., 1996). These proteins are also secreted within the developing peripheral nerve trunk, spinal cord, optic nerve, neural-crest cells and trigeminal ganglion of the mouse embryo (E13.5-E18.5) (Gruenbaum-cohen et al., 2009; Fincham et al., 1984), and

appear to be localized primarily to nerve fibre-like structures, suggesting that they might have a role in nervous system development and nerve cell differentiation, as observed here (Gruenbaum-cohen et al., 2009; Fincham et al., 1984).

The present results also demonstrated that PDL cells cultured in GDM alone and GDM with TRAP exhibited morphologies similar to astrocytes, found in both the CNS and PNS, and to Schwann cells, found exclusively in PNS (Maeda et al., 1999). Notably, the PDL cells did not exhibit the CNS-associated oligodendrocyte-like morphology in either the control cultures or the TRAP-induced cultures after 7 days, in contrast to the observations reported in Chapter 3 that the PDL cells cultured in GDM alone for 10 days exhibited all three types of glial cell-like morphology, including astrocytes, Schwann cells and oligodendrocytes. The reason for this discrepancy is not yet clear, but it may partly be due to differences in the length of time the PDL cells were cultured in GDM (in the absence or presence of TRAP) with 10 days of culture in GDM possibly being necessary for the development of oligodendrocyte-like morphology, as shown in Chapter 3.

It has previously been shown that Schwann cells play an indispensible role in peripheral nerve development and regeneration, mainly by producing/secreting neural growth factors (NGF, BDNF, NT-4/5), surface adhesion molecules and forming a complex laminin-, fibronectin-, heparan sulphate- and tenascin-rich basement membrane for nerve cells. Thus, the TRAP-induced Schwann cell formation observed here *in vitro* may be at least partly responsible for the TRAP peptide-mediated nerve regeneration observed here (Yuan et al., 2007; Frostick et al., 1998; Rodriguez et al., 2000). This is consistent with the other observation that elevated levels of amelogenin gene products are present within the sub-cortical region of the brain containing a dense glial cell population, again suggesting the possible role of such proteins in glial cell development/differentiation (Gruenbaum-cohen et al., 2009).

In summary, the present study showed for the first time that the amelogenin isoform TRAP appears to promote the differentiation of PDL cells to nerve-like cells and to PNS-associated glial cells, whereas the higher molecular weight EMD components ablated nerve and glial cell formation.

Chapter 8

8.1. Introduction

Stem cells can be defined as having the ability to give rise to at least one differentiated cell type as well as the capacity to self-renew (Collins et al., 2005; Lemoli et al., 2005). In contrast to the large majority of the cell population of adult tissues that are committed to a specific function, stem cells are uncommitted until they receive signals to differentiate into progenitor/precursor cells before forming fully differentiated and functionally active cells in culture *in vitro* and in the tissue microenvironment *in vivo* (Weissman et al., 2000; Lemoli et al., 2005). The stimuli for cell differentiation are therefore critical for tissue homeostasis, repair and replenishment (Weissman et al., 2000; Till and McCulloch, 1961; De Miguel et al., 2010). For example, mouse embryonic cells have been shown to require external factors such as recombinant leukemia inhibitory factor (LIF), fibroblast growth factors (FGF) and bone morphogenetic proteins (BMP) to maintain their plasticity and capacity to self-renew in culture (Chen et al., 2008; Niwa et al., 1998; Smith et al., 2001; Ying et al., 2003; De Miguel et al., 2010). Similarly, recombinant BMP-2, Wnt3a, Wnt5a and insulin-like growth factor (IGF) have been shown to stimulate a range of differentiation pathways of precursor/stem cells, including osteogenesis, gliogenesis and myogenesis (Baksh et al., 2007; Singhatanadgit et al., 2008; Kasai et al., 2005; Husmann et al., 1996). Such growth mediators can induce cell differentiation by binding to specific cell surface transmembrane receptors to form ligand-receptor complexes (Seto et al., 2002; Caron et al., 2001; Conner and Schmid, 2003). These activate intracellular second messengers, often involving a cascade of protein kinase intermediaries (Caron et al., 2001; Conner and Schmid, 2003), which ultimately translocate to the nucleus and modulate target genes, thus eliciting the biological response to the ligand (Barnes et al., 1999; Johnson and Vaillancourt, 1994). For example, the BMP-2 and 4 ligands bind specifically to the BMP receptors (BMPR) I and II, the resultant BMP2/4-BMPR complex then activating second messengers including Smad4 and Smad1/5/8 which translocate to the nucleus and up-regulate osteogenic transcription factor genes such as Runx2 (Ryoo and Lee, 2006; Singhatanadgit et al., 2006).

Receptor-mediated endocytosis is generally considered to be the main mechanism by which ligand-receptor complexes are internalized into the cells, first into early endosomes and then transported to other organelles including the trans-Golgi network (TGN) and lysosomes.

Dissociation of the ligands from the receptors usually occurs in the acidic environment of these organelles, the receptors often being recycled back to the plasma membrane (Seto et al., 2002) and the ligands undergoing hydrolytic breakdown in the lysosomes (French et al., 1995; Seto et al., 2002). Receptor-mediated endocytosis is generally considered a main mechanism by which cells regulate the availability of cell surface receptors and thereby the response to ligand (French et al., 1995; Seto et al., 2002). A number of studies have provided evidence for the importance of receptor-mediated endocytosis in growth factor-mediated signal transduction, including Wnt (Baksh et al., 2007), BMP (Singhatanadgit et al., 2006; Jortikka et al., 1997), FGF (Citores et al., 2001), IGF (Furlanetto, 1998) and VEGF (Wang et al., 2002).

In a number of studies of the mouse amelogenins in tooth development, the full-length amelogenin (M180) and its isoforms [A-4](M50) and [A+4] (M73) have also been reported to bind to a protein which is sometimes located at the cell surface, the lysosomal-associated membrane protein (LAMP)-1), in mouse embryonic dental follicle cells involved in dentin, cementum and PDL formation (Ten cate et al., 1996; Heritier et al., 1982; Zhang et al., 2010; Tompkins et al., 2006; Iacob et al., 2008). Additionally, the amelogenin peptides were shown to be transported to intracellular vesicles including early endosomes and lysosomes (Iacob and Veis, 2008; Tompkins et al., 2006; Xu et al., 2008). Similarly, a previous study has also suggested that at least some component(s) present in EMD may also be internalized into cells by receptor-mediated endocytosis (Reseland et al., 2006). Thus, EMD was found to internalize into osteoblasts via clathrin-coated pits, identified by co-immunolabelling of anti-EMD antibody and anti-AP-2, a clathrin-associated protein (Reseland et al., 2006). However, the most crucial initial process of EMD binding to the osteoblast cell membrane and the intracellular transport and fate of EMD were not examined in this study (Reseland et al., 2006). As noted in Chapter 1, EMD is a complex mixture of various proteins including amelogenins, sheathlins and enamelins (Brookes et al., 1995; Hu et al., 1997). Thus, the specificity of the antibody raised against EMD and the specific component(s) internalized into the osteoblasts remain unclear in this report (Reseland et al., 2006). As noted above, receptor-mediated endocytosis is the main mechanism by which soluble ligands bind and internalize into the cell, but whether commercially prepared EMD and the EMD component(s) bind to the cell membrane and internalize via receptor-mediated endocytosis is not yet clear.

During porcine development EMP has been shown to be secreted by specialized epithelial cells (ameloblasts) via unique extracellular projections (tome's processes) (ten Cate

et al., 1996; Heritier et al., 1982) and to precipitate on the cell surface, leading to the development of tooth-associated tissues (Deutsch et al., 1995). Using Scanning Electron Microscopy (SEM) it was observed that the commercial preparation EMD precipitates on human PDL tissue sections *ex vivo* and forms spheres or short rod-like structures (Cattaneo et al., 2003). In addition, EMD has also been found to precipitate on the surface of PDL cells *in vitro* and to stimulate cell proliferation, ECM production and osteogenic differentiation (Cattaneo et al., 2003; Gestrelius et al., 1997; Zeichner et al., 2006). It has therefore been suggested that such insoluble complexes of the full-length hydrophobic amelogenin protein, a main component of EMD, may be at least partially responsible for these cellular activities by PDL cells (Cattaneo et al., 2003; Gestrelius et al., 1997; Zeichner et al., 2006), in addition to the possible responses resulting from the binding and internalization of soluble EMP component(s) by receptor-mediated endocytosis (Iacob and Veis, 2008; Tompkins et al., 2006; Xu et al., 2008).

However, in contrast to 'soluble' ligands, internalization of such insoluble particulate matter, including also certain viruses, bacteria, bacterial toxins and macromolecular complexes such as collagen, occurs via phagocytosis (Groves et al., 2008). In mammalian cells, this process is carried out primarily by the cells of the immune system (e.g. macrophages, dendritic cells, monocytes and neutrophils) and involves specialized intracellular vacuoles (phagosomes) in which 'insoluble' macromolecular substances are degraded (Greenberg et al., 1995; Groves et al., 2008). Although phagocytosis has also been shown to occur via receptors (e.g. FcγRs, complement receptor (CR)3) which elicit development of submembranous actin filament assemblies and pseudopod extensions (Lowell, 2006; Greenburg, 1995) via intracellular signal transduction processes and to be ultimately responsible for uptake of particulate matter (Lowell, 2006), they are unlikely to have a role in cell differentiation (Lowell, 2006; Greenburg, 1995).

In the present study it has been shown that the components of EMD differentially regulate a range of PDL cell differentiation activities, including osteogenesis, adipogenesis, chondrogenesis, angiogenesis, vasculogenesis, neurogenesis and gliogenesis *in vitro* (Chapters 4-7). However, the interaction of (commercial heat-treated preparations) EMD and the various components of EMD with PDL cells is not yet known. Experiments were therefore carried out to determine: (i) whether EMD and the EMD Fractions (C and A) bind to PDL cells, (ii) whether EMD and the EMD Fractions are internalized into the cells, and (iii) intracellular localization/transport of EMD and the EMD Fractions.

8.2. Materials and methods

PDL cell culture and SEM were performed as described in Chapter 2; additional methods used in this chapter are described below.

8.2.1. Binding and intracellular uptake of EMD and the EMD Fractions by PDL cells

To assess cell binding, PDL cells were seeded onto 24-well plates on glass coverslips at a density of 10^4 cells/well and cultured in GM for 2 days. The medium was replaced with cold GM containing 30 μg/ml of EMD, Fraction C and Fraction A which had been directly biotin-labelled (provided by Institut Straumann, Basel, Switzerland) and the cells maintained at 4°C for 45 min, washed 3 x with cold PBS, fixed with 4% PFA for 15 min, reacted with Alexa Fluor-streptavidin (Invitrogen, Paisley, UK) for 30 min and visualized under the fluorescence microscope. To determine whether the EMD and the EMD Fractions were membrane-associated, replicate cultures were washed 3 x with cold PBS, treated with trypsin-EDTA at 37°C for 5 min and the detached cells centrifuged at 1500 rpm for 5 min. The cell pellets were washed and resuspended in PBS, fixed with 4% PFA for 15 min, reacted with Alexa Fluor-streptavidin for 30 min, air dried on a glass slide and coverslips with mounting medium were placed on the cells.

To examine whether EMD and the EMD Fractions internalized after the initial incubation at 4°C as above, the cells were washed 3 x with PBS, incubated with GM at 37°C for 3 and 6 h, fixed with 4% PFA, permeabilised using 0.1% Triton-X for 10 min and reacted with Alexa Fluor-streptavidin (Invitrogen) for 30 min. To eliminate the possibility that any positive staining (i.e. red fluorescence) might be due to membrane bound/extracellular ligand, replicate cultures were treated with trypsin-EDTA for 5 min prior to fixation with PFA. The detached cells were centrifuged at 1500 rpm for 5 min, re-cultured for a further period of 3 h to allow the cells to adhere/spread for visualizing cellular morphology, fixed with 4% PFA, permeabilised using 0.1% Triton-X for 10 min and reacted with Alexa Fluor-streptavidin (Invitrogen) for 30 min. Nuclei were stained blue using Hoechst dye.

8.2.2. Intracellular localization EMD and the EMD Fractions

To examine intracellular localization of EMD and the EMD Fractions after binding, the biotinylated-EMD, Fraction C and Fraction A were co-localized with markers of the trans-Golgi network (TGN) and lysosomes using specific antibodies as described below. The cells were seeded and cultured on 24-well plates on glass coverslips for 2 days and then incubated with the biotin-labelled ligands at 4°C for 45 min, as described above. The cells were then washed 3 x with cold PBS, incubated with warm GM at 37°C for 45 min and 3 h, fixed with 4% PFA and permeabilised using 0.1% Triton-X for 10 min. These conditions were used because a number of ligands (mouse amelogenin isoform [A-4/A+4], Wnt, Basolateral proteins, STxB (non-toxic β-subunit of shiga toxin)) have previously been shown to be transported to the TGN after 30 to 60 min and to the lysosomes after 2 to 6 h of incubation at 37°C following binding (Iacob and Veis, 2008; Cancino et al., 2007; Port and Basler, 2010; Mallard et al., 2002). The samples were immunostained for 58K, a marker of the TGN localized in the perinuclear region (Bashour et al., 1998), and LAMP-1, a membrane protein primarily associated with lysosomal organelles (Chen et al., 1985), as described previously (Singhatanadgit et al., 2008). After treating with a blocking solution containing 10% normal goat serum (NGS) in PBS for 1 h, the cells were incubated for 1 h at RT with the primary mouse monoclonal anti-58K (Sigma) (for the samples incubated at 37°C for 45 min) and anti-LAMP-1 (Abcam) (for the samples incubated at 37°C for 3 h) antibodies diluted 1:200 and 1:20, respectively, in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor 488 secondary antibody (green fluorescent) (Invitrogen) diluted 1:200 in PBS containing 1% NGS for 1 h at RT. Cells treated with non-specific mouse IgG were used a control. The TGN was visualized by perinuclear green fluorescent staining after treatment with anti-58K and lysosomal vesicles were visualized by punctate green fluorescent staining after treatment with LAMP-1. The cells were then further reacted with Alexa Fluor-streptavidin 594 (red fluorescent) (Invitrogen, Paisley, UK) for 30 min to visualize the biotin-labelled EMD and the EMD Fractions; nuclei were stained blue using Hoechst dye.

Whether in lysosomes at 3 h, as indicated by the results of fluorescence microscopy, replicate cultures were prepared for immunogold electron microscopy (IEM) to elucidate localization of EMD, Fraction C and Fraction A at the ultrastructural level, as follows. The cell cultures were maintained at 4°C for 45 min with GM in the presence of biotinylated-EMD, Fraction C and Fraction A, washed 3 x with cold PBS and incubated with warm GM at 37°C for 3 h. The cells then detached by incubating in Trypsin-EDTA for 5 min, after which

they were centrifuged at 1500 rpm for 5 min and the cell pellets fixed with 0.5% glutaraldehyde at 4°C for 30 min, dehydrated in cold ethanol and embedded at -20°C in LR White resin (Agar Scientific, Stansted, UK). Ultra-thin sections (approximately 0.1 μm thick) were collected on specimen grids (Agar Scientific), rehydrated with distilled water and then were incubated with 10% NGS in PBS for 1 h to block non-specific binding, then washed 3 x washes with PBS. The sections were subsequently incubated with mouse-monoclonal LAMP-1 antibody (1:20 in PBS containing 1% NGS) for 1 h at RT. Non-specific mouse IgG was used as the control for the primary antibody LAMP-1. After 3 x washes with PBS, the sections were incubated with 5 nm gold-conjugated goat antibody (Agar Scientific) (diluted 1:200 in 1% NGS in PBS) for 1 h at RT, then treated with 20 nm gold-conjugated streptavidin for 30 min to visualize the biotinylated EMD and the EMD Fractions. Sections not treated with biotin-labelled EMD and the EMD Fractions were also incubated with 20 nm gold-labelled streptavidin as a specificity control. The sections were stained with uranyl acetate (1 min) and lead citrate (1 min) to enhance the contrast between intracellular structures of differing electron density and the presence of LAMP-1 indicated by small, round black gold particles (5 nm in size) and the presence of EMD, Fraction C and Fraction A indicated by the 20 nm round gold particles. The sections were examined under a TEM (JEOL 100 CX II; Jeol Ltd, Welwyn Garden City, UK).

8.2.3. Phase-contrast and SEM analysis of EMD, Fraction C and Fraction A interaction with PDL cells

EMD has previously been shown to precipitate and form spheres and short rod-like structures on the PDL cell surface *in vitro* and on PDL tissue section *ex vivo* and it has been suggested that the proline-rich full-length hydrophobic amelogenin protein, a main component of EMD, is likely to be responsible for the formation of such structures (Cattaneo et al., 2003; Gestrelius et al., 1997; Zeichner et al., 2006). To examine whether Fraction C and Fraction A (sub-fractions of EMD which contain mainly amelogenin and/or amelogenin isoforms (Chapter 1)) also precipitate on the PDL cell surface, 10^4 cells/well were seeded onto coverslips in 24-well plates and cultured for 2-3 days in GM. EMD, Fraction C and Fraction A (all at 30 μg/ml) were then added and the cells incubated for 24 h at 37°C after which they were visualized under phase-contrast microscope to examine the possible formation of insoluble particulate material. Replicate cultures were also fixed in 1% glutaraldehyde at 4°C

for 30 min and prepared for SEM analysis. PDL cells without any treatment with EMD or the EMD Fractions were used as a control. To examine whether EMD, Fraction C and Fraction A in GM precipitate on tissue culture plastic in the absence of any cells, 30 μg/ml EMD and the EMD Fractions were added in GM on cover slips in 24-well plates and incubated for 24 h at 37°C, after which they were fixed in 1% glutaraldehyde at 4°C for 30 min and prepared for SEM analysis.

8.3. Results

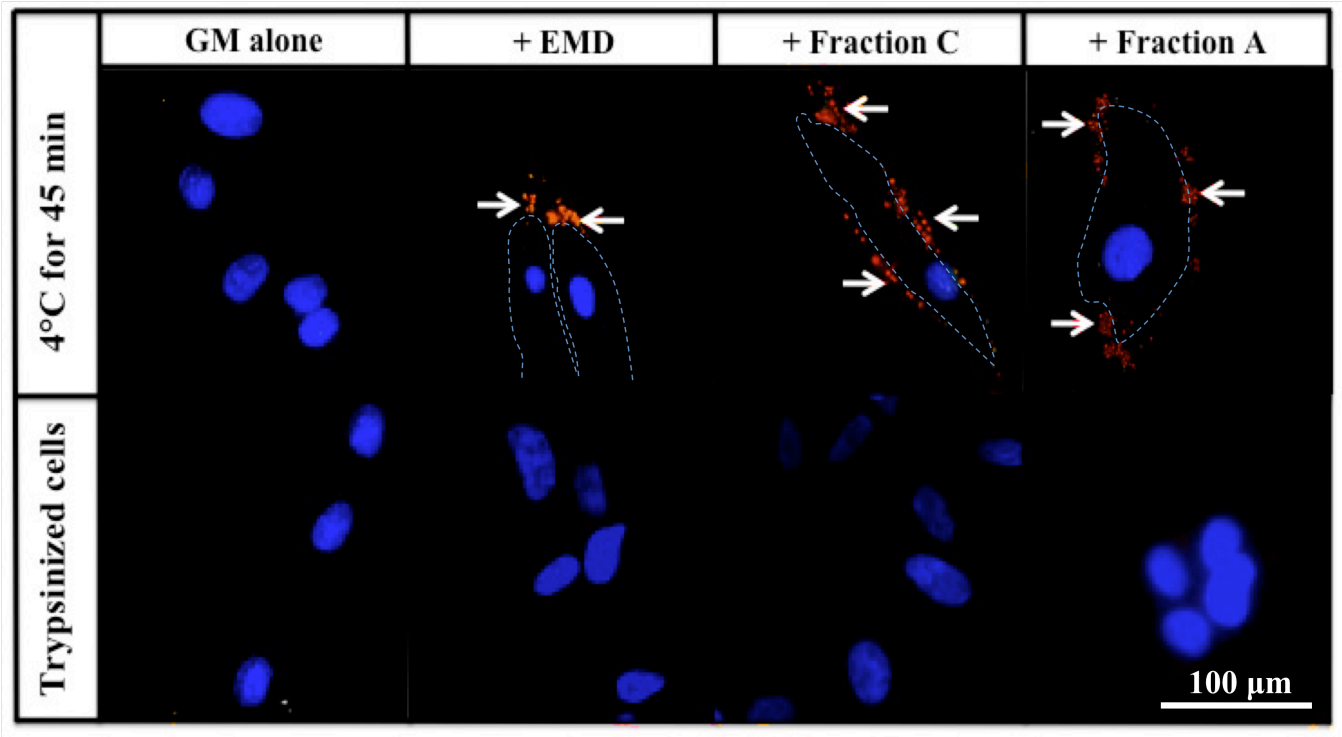
8.3.1. Binding and uptake of EMD and the EMD Fractions by PDL cells

To determine whether EMD and the EMD Fractions bind to the PDL cells, the cells were incubated for 45 min with biotinylated EMD, Fraction C and Fraction A at 4°C, at which temperature there is no uptake of exogenous ligand (Seto et al., 2002; Conner and Schmid, 2003; Iacob et al., 2008). The results in Figure 8.1 show that, under these conditions, red fluorescent staining corresponding to EMD, Fraction C and Fraction A was observed to be localized at the cell membrane with little if any fluorescence present intracellularly. To establish whether the red fluorescent staining of EMD, Fraction C and Fraction A was extracellular/associated with the cell membrane, the cells which had been incubated with EMD and the EMD Fractions at 4°C for 45 min were then treated with trypsin-EDTA, centrifuged, fixed, reacted with streptavidin-Alexa Fluor 594 and air dried on glass slides. Figure 8.1 shows that after treatment with trypsin-EDTA there was no red fluorescent staining associated with any of the cells, indicating that EMD and the EMD Fractions were removed/digested by the trypsin/EDTA treatment and were therefore likely to have been entirely membrane-bound after incubation at 4°C.

When the cultures were incubated with the biotinylated EMD and the EMD Fractions for 45 min at 4°C, washed and re-cultured at 37°C for 3 h, the red fluorescence corresponding to EMD and the EMD Fractions was found to be localized intracellularly, with a diffuse cytosolic distribution after 3 h, as shown in Figure 8.2. Moreover, after the cells were re-cultured for 6 h at 37°C, the red fluorescence was found to be intracellular, but the fluorescent staining was localized mainly within few large, round intracellular vesicle-like structures (generally < 10). To establish whether the visualized EMD and the EMD Fractions were intracellular and not on the cell membrane, the cells were first incubated with biotinylated-EMD and the EMD Fractions at 4°C for 45 min, washed, cultured for a further period of 3 h at 37°C, treated with trypsin-EDTA as before in order to digest any extracellular EMD/Fractions-ligands and then re-cultured for 3 h (total of 6 h of culture at 37°C). The results in Figure 8.2 show that the red fluorescent staining corresponding to EMD, Fraction C and Fraction A was still clearly evident (within large, round vesicle-like structures) even after trypsinization, indicating that EMD and the EMD Fractions were likely to have been internalized after incubation at 37°C for 3 and 6 h. Manual counting of the cells exhibiting

intracellular red fluorescent staining after 6 h showed that 8.5, 6.6 and 12.3% to the total PDL cells internalized EMD, Fration C and Fraction A, respectively (Appendix Material 3.1).

Figure 8.1. Micrographs of biotinylated EMD, Fraction C and Fraction A binding to PDL cells. Cells were cultured in the absence and presence of EMD and the EMD Fractions at 4°C for 45 min, washed, fixed and reacted with red fluorescent Alexa Fluor-streptavidin. Replicate cultures were washed, trypsinized, centrifuged, fixed, reacted with red fluorescent Alexa Flour-streptavidin and air-dried on glass slide. EMD and the EMD Fractions are visualized by red fluorescent staining. The nuclei are stained blue using Hoechst dye. White arrows show cell-associated EMD, Fraction C and Fraction A. Magnification x 60.



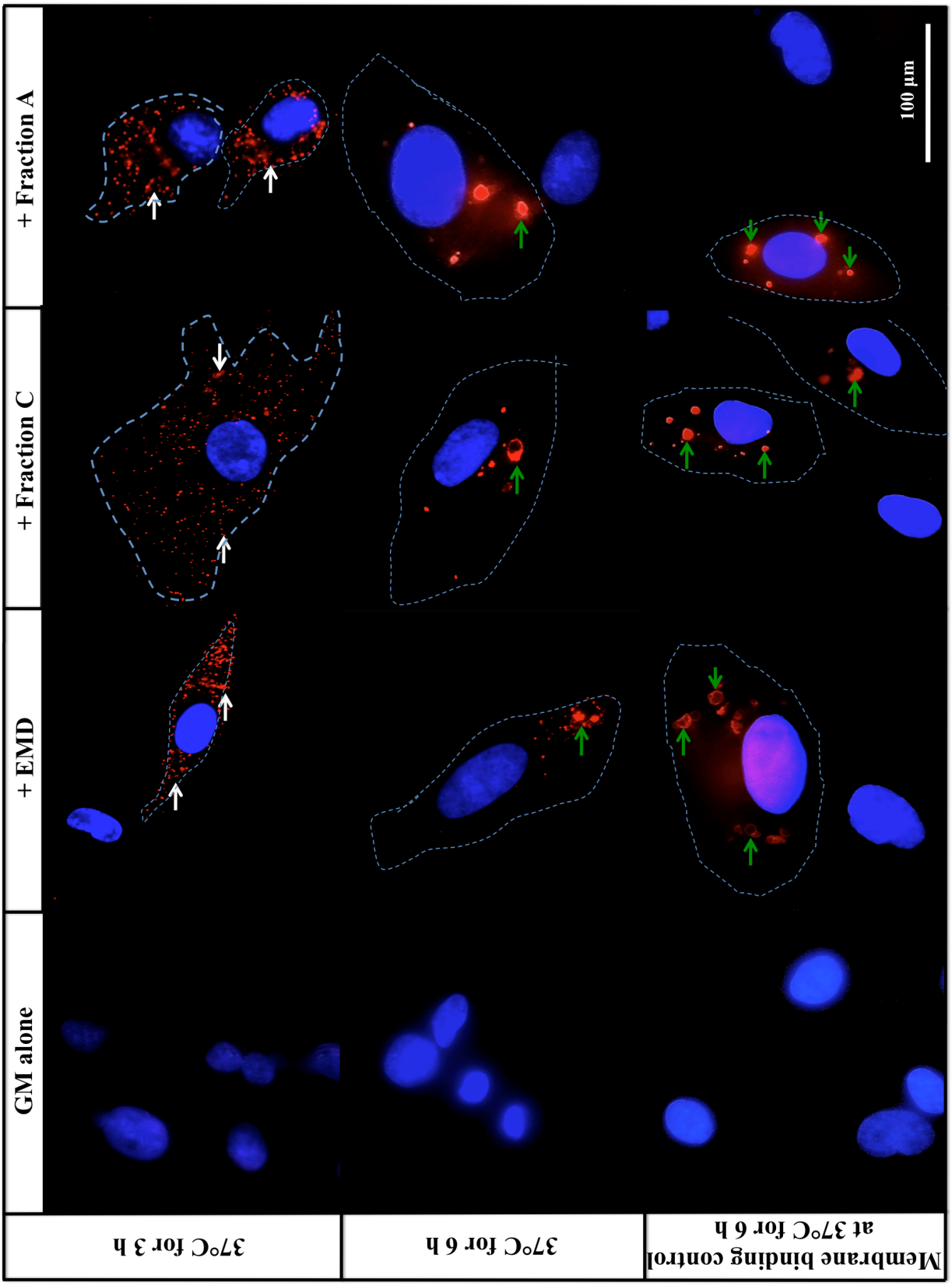

Membrane binding control at 37°C for 6 h
37°C for 6 h
37°C for 3 h
GM alone
+ EMD
+ Fraction C
+ Fraction A
100 µm

Figure 8.2. Micrographs of the internalization of biotinylated EMD, Fraction C and Fraction A by PDL cells. Cells were incubated at 4°C for 45 min in the absence and presence of EMD, Fraction C and Fraction A, then washed and further incubated at 37°C for 2 and 6 h, washed, fixed and reacted with Alexa Fluor-streptavidin. Replicate cultures were trypsinized to digest membrane-bound ligands and further cultured for 3 h to allow the cells to re-attach, then reacted with red fluorescent Alexa Fluor-streptavidin. EMD and the EMD Fractions are visualized by red fluorescent staining in the PDL cells. Note the white arrows showing the diffuse distribution of the red fluorescent EMD and the EMD Fractions. Green arrows show the localization of EMD and the EMD Fractions within distinct intracellular vesicle-like structures. The nuclei are stained blue using Hoechst dye. Magnification x 60.

8.3.2. Intracellular localization of EMD and the EMD Fractions in PDL cells

The intracellular localization of biotinylated-EMD and the EMD Fractions was examined by co-labelling with an intracellular marker of the TGN and of the lysosomes, 58K and LAMP-1, respectively, as described in the Materials and methods. Figure 8.3 shows that in the control cultures which were not incubated with EMD and the EMD Fractions there was no red fluorescent staining, while 58K and LAMP-1 staining was observed by the peri-nuclear green fluorescence of the 58K antigen and the punctate green fluorescence of the LAMP-1 (lysosomal) antigen, respectively, as shown previously (Tajika et al., 2004; Bashour et al., 1998; Chen et al., 1985). It is notable that there were a large number (generally > 50) of LAMP-1 positive diffuse structures, most probably lysosomes, as previously reported (Chen et al., 1985; Iacob et al., 2008) (Figure 8.3). No green fluorescent staining was detected in the negative control in which sections were reacted with the non-specific mouse IgG antibody.

When biotinylated-EMD and the EMD Fractions were incubated with PDL cells at 4°C for 45 min, washed, re-cultured in GM at 37°C for 45 min, stained for TGN antigen 58K (Alexa Fluor 488; green fluorescent) and then reacted with streptavidin-Alexa Fluor 594, the red fluorescent staining corresponding to EMD and the EMD Fractions was found to co-localize with the peri-nuclear green fluorescence of the TGN, as shown in Figure 8.3. When the incubation of the PDL cells was extended for 3 h at 37°C, EMD, Fraction C and Fraction A were found to be localized with LAMP-1-positive green fluorescent diffuse structures, presumably lysosomes, as shown in Figure 8.3. Similarly, when biotinylated-chemically synthesized TRAP peptide was incubated with PDL cells at 4°C for 45 min, washed and reacted with streptavidin-Alexa Fluor 594, red fluorescent staining corresponding to TRAP was found to be extracellular and possibly membrane-bound, as shown in Figure 8.4. After 45 min of incubation at 4°C, when replicate cultures were washed and further incubated in GM at 37°C for 45 min the red fluorescent staining was found to co-localize with the peri-nuclear green fluorescence of TGN (Fig. 8.4). After 3 h at 37°C, the red fluorescent staining was found to co-localize with the diffuse green fluorescent staining of LAMP-1, demonstrating that the intracellular fate of the chemically synthesized biotinylated-TRAP was similar to that of the EMD Fractions.

To establish whether EMD and the EMD components are in lysosomes at 3 h, as indicated by the results of fluorescence microscopy, the precise ultrastructural localisation of

the internalized biotinylated EMD, Fraction C and Fraction A was examined using IEM by reacting sections of the cells with streptavidin-gold (20 nm particles) and anti-LAMP-1 (5 nm gold particles), as described in **Materials and methods**. The results in Figure 8.5 show that in the control sections which were not treated with EMD and the EMD Fractions, no 20 nm gold particles were detected, whereas the 5 nm gold particles corresponding to LAMP-1 appeared to localize in sub-cellular organelles, presumably lysosomes. No gold particles were detected in the negative control in which sections were reacted with the non-specific mouse IgG antibody (Appendix Material 3.2). In the sections incubated with EMD, Fraction C and Fraction A, 20 nm gold particles were observed to be co-localized with the 5 nm gold particles corresponding to the LAMP-1 antibody (Figs. 8.6, 8.7 and 8.8).

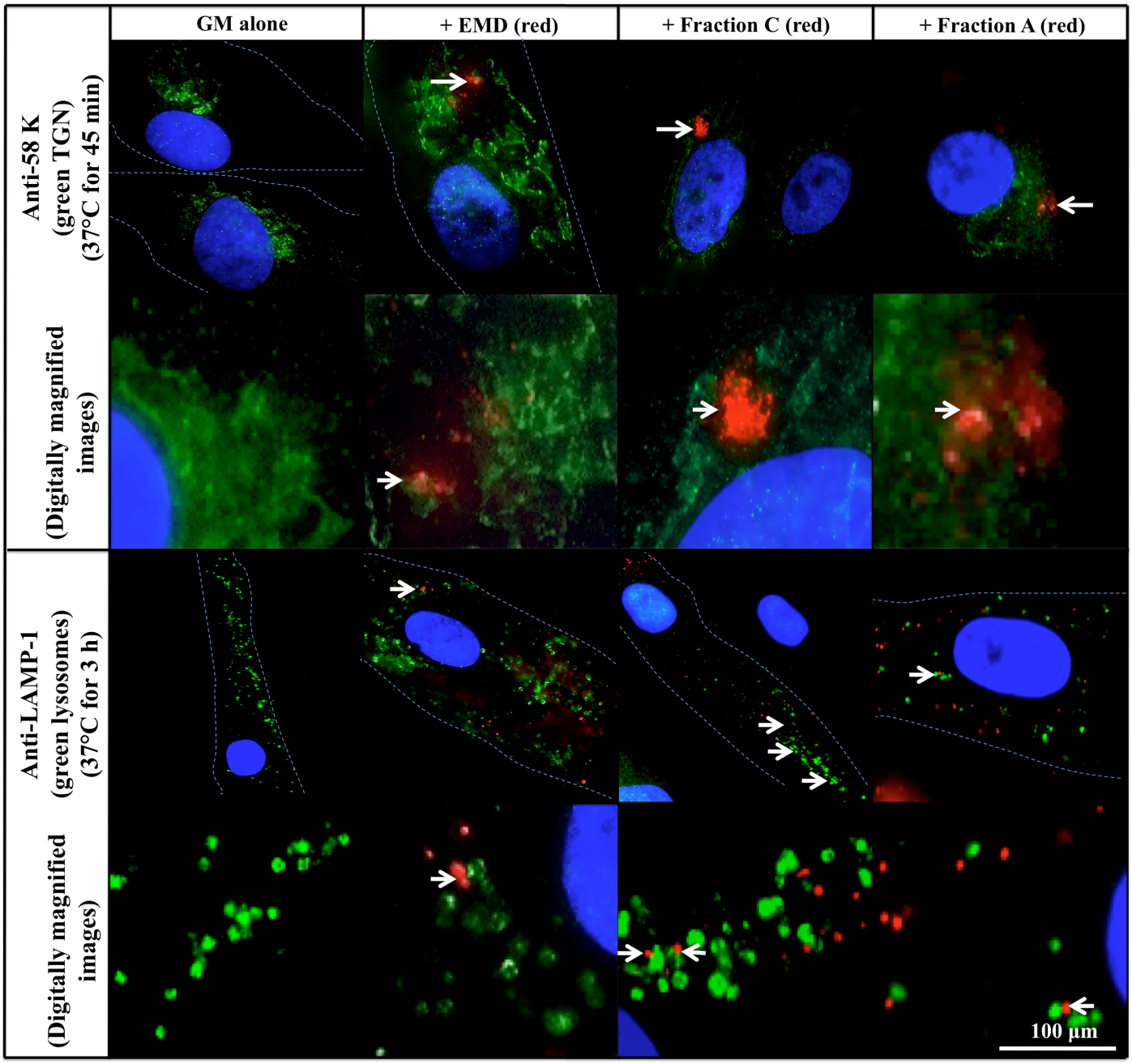


Figure 8.3. Micrographs of co-labelled PDL cells, incubated with biotinylated EMD, Fraction C and Fraction A (observed by red fluorescent staining) and immunostained for either (A) TNG marker 58K or (B) lysosome marker LAMP-1 (both observed by green fluorescent staining). PDL cells were cultured in the absence and presence of EMD and the EMD Fractions at 4°C for 45 min, washed and re-cultured at 37°C for 45 min (A) or 3 h (B). White arrows show co-localization of EMD and the EMD Fractions with 58K-positive TGN (A) and LAMP-1-positive structures (B). Nuclei are stained blue using Hoechst dye. Magnification x 100.

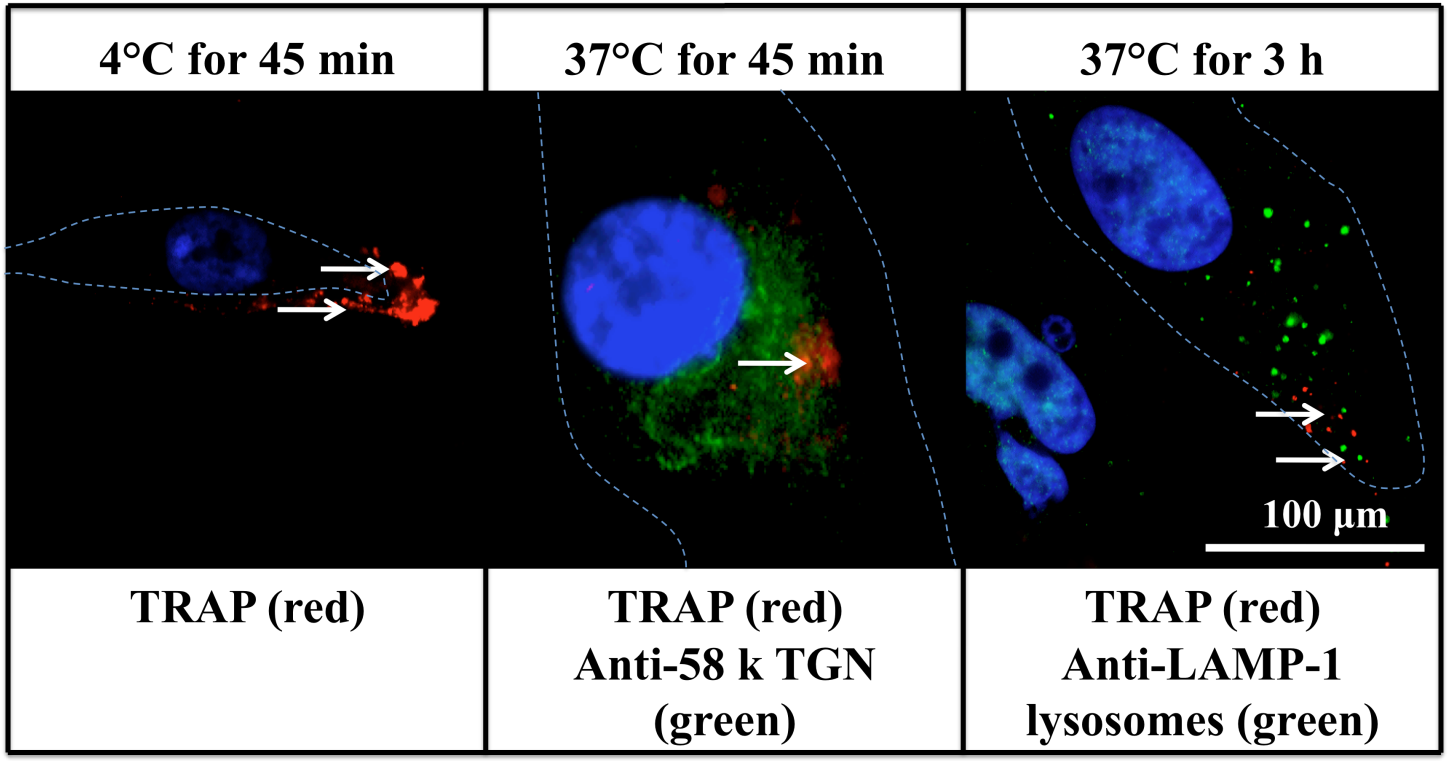


Figure 8.4. Micrographs of PDL cells incubated with biotinylated TRAP (red fluorescent) and co-labelled for intracellular vesicles (TGN and lysosomes; green fluorescence). PDL cells were cultured in the absence and presence of TRAP at 4°C for 45 min, washed and further incubated in GM at 37°C for 45 min and 3 h. White arrows show the co-localization of TRAP with 58K-positive TGN and LAMP-1-positive lysosomes-like structures. White boxed area shows magnified micrographs of co-localized TRAP with 58K-positive and LAMP-1-positive structures. The nuclei are stained blue using Hoechst dye. Magnification x 60 (4°C for 45 min) and x 100 (37°C for 45 min and 3 h).

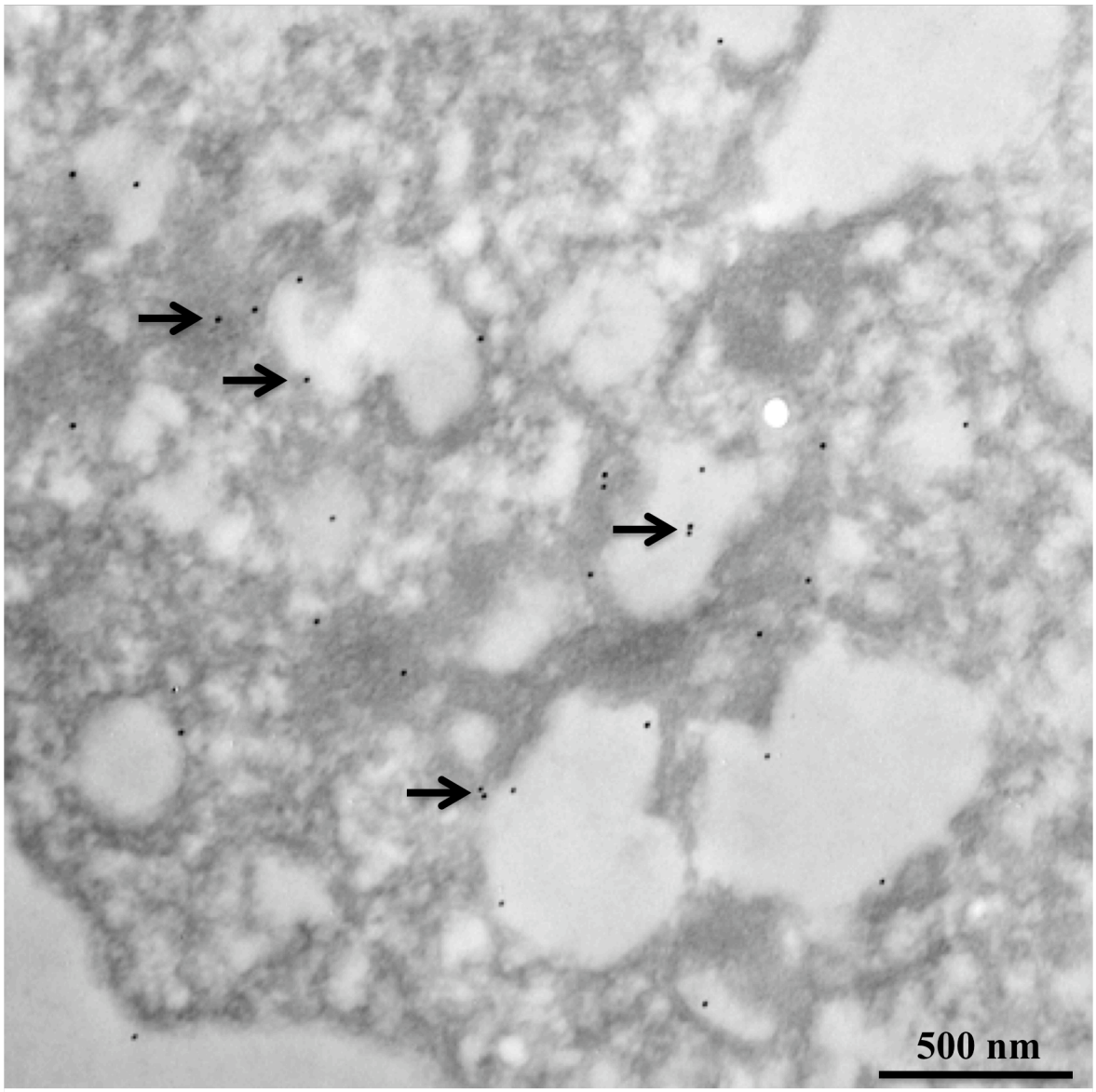


Figure 8.5. Control immunogold EM section demonstrating the ultrastructure of a PDL cell cultured in GM in the absence of biotinylated EMD and the EMD Fractions. The sections reacted with mouse monoclonal LAMP-1 antibody and reacted with 5 nm gold-conjugated goat antibody (secondary antibody). In order to examine whether 20 nm gold-conjugated-streptavidin reacts with the cells in the absence of biotinylated EMD and the EMD Fractions, the sections were also reacted with 20 nm gold-conjugated-streptavidin (control). Black arrows show 5 nm gold particles most probably localized within the lysosomes. Note the absence of 20 nm gold particles.

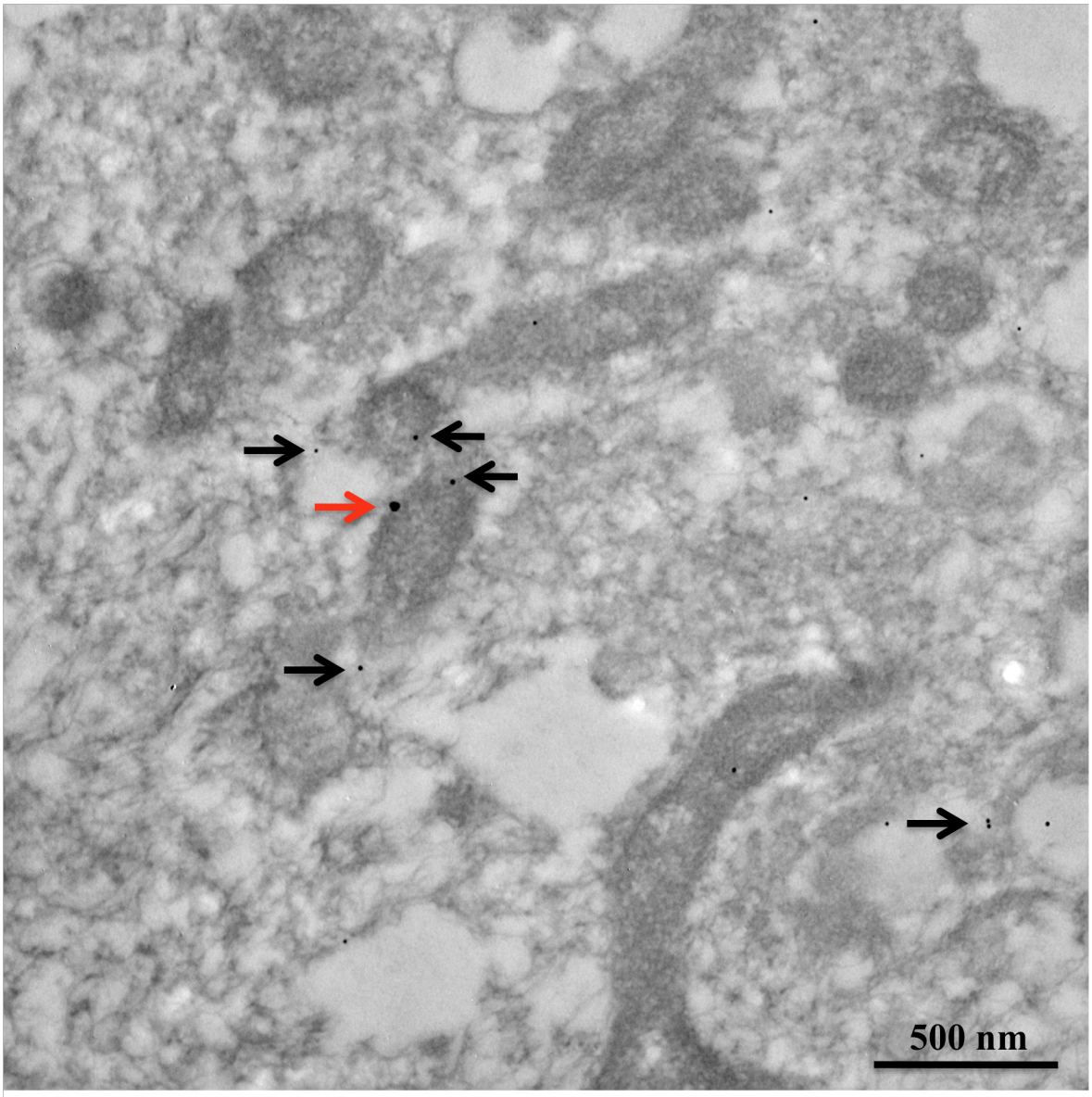


Figure 8.6. Double-labelling Immunogold EM demonstrating the ultrastructure of a PDL cell cultured in GM in the presence of biotinylated EMD for 3 h at 37°C. The sections were stained for LAMP-1 and reacted with 5 nm gold-conjugated goat antibody (secondary antibody) and 20 nm gold-conjugated-streptavidin. Black arrows show 5 nm gold particles corresponding to LAMP-1 that are most probably localized within the lysosomes and a red arrow shows a 20 nm gold particle corresponding to biotinylated EMD. Note the presence of EMD apparently closely associated with LAMP-1.

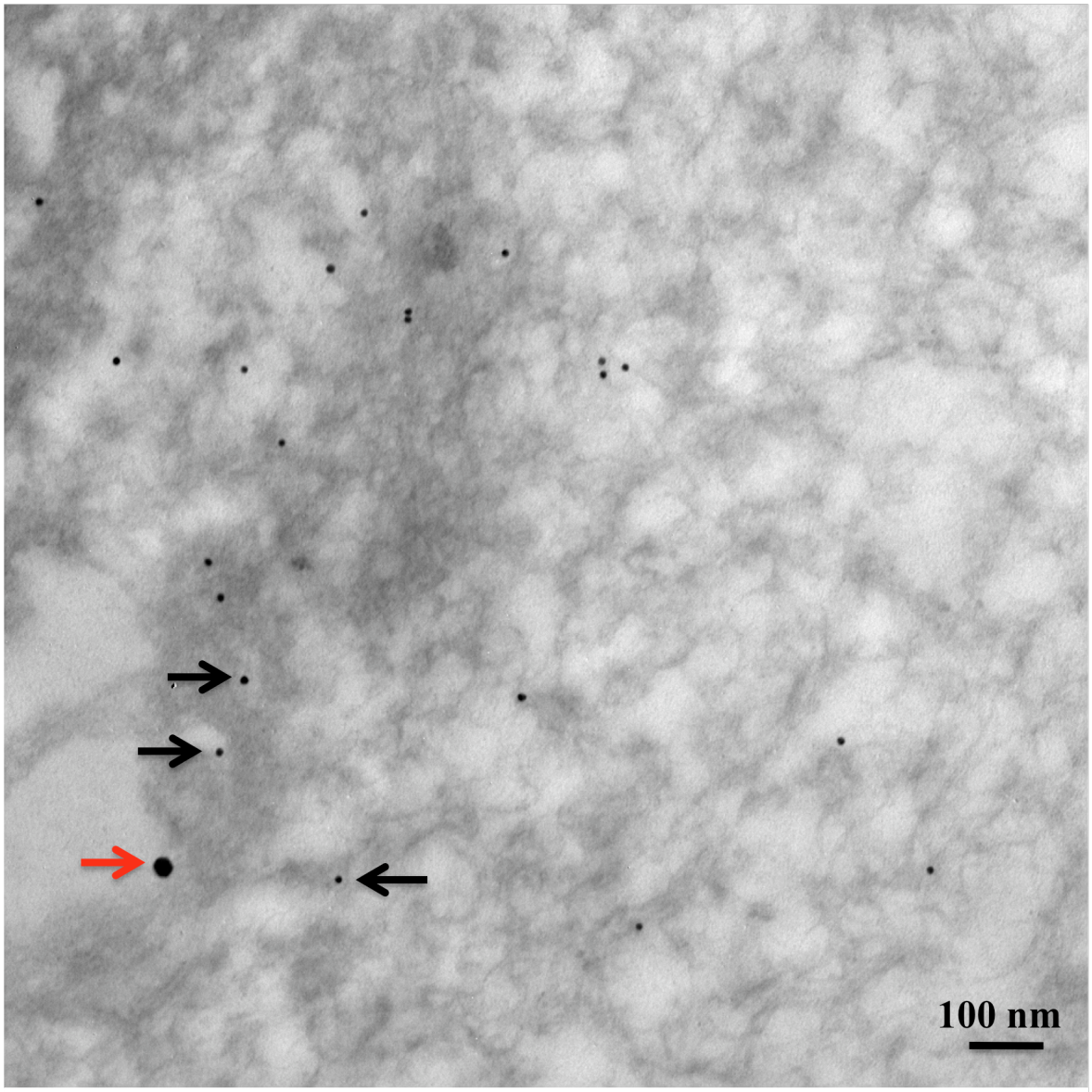


Figure 8.7. Double-labelling Immunogold EM demonstrating the ultrastructure of a PDL cell cultured in GM in the presence of biotinylated Fraction C for 3 h at 37°C. The sections were stained for LAMP-1 and reacted with 5 nm gold-conjugated goat antibody (secondary antibody) and 20 nm gold-conjugated-streptavidin. Black arrows show 5 nm gold particles corresponding to LAMP-1 that are most probably localized within the lysosomes and a red arrow shows a 20 nm gold particle corresponding to biotinylated Fraction C. Note the presence of Fraction C apparently closely associated with LAMP-1.

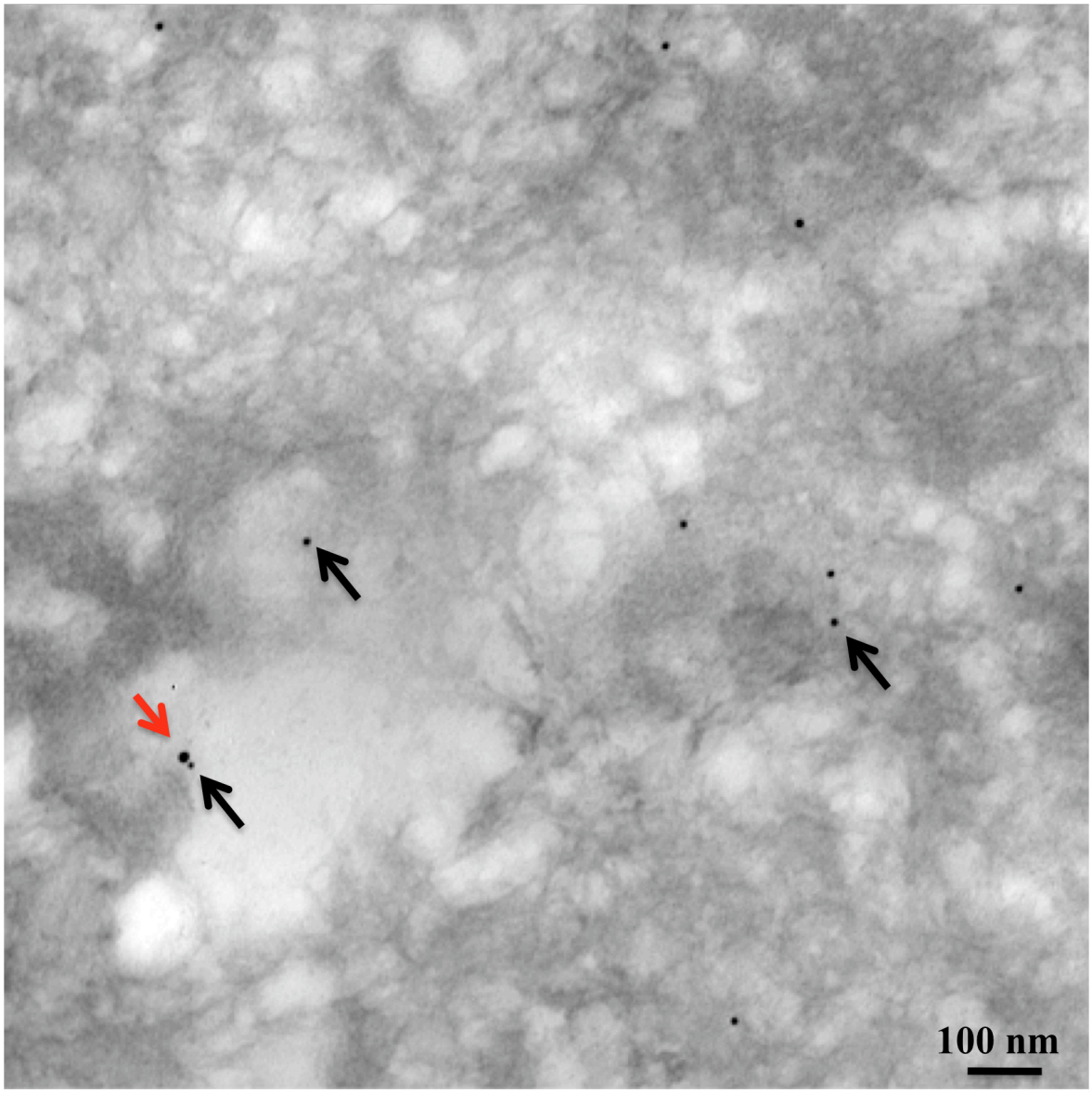


Figure 8.8. Double-labelling Immunogold EM demonstrating the ultrastructure of a PDL cell cultured in GM in the presence of biotinylated Fraction A for 3 h at 37°C. The sections were stained for LAMP-1 and reacted with 5 nm gold-conjugated goat antibody (secondary antibody) and 20 nm gold-conjugated-streptavidin. Black arrows show 5 nm gold particles corresponding to LAMP-1 that are most probably localized within the lysosomes and a red arrow shows a 20 nm gold particle corresponding to biotinylated Fraction A. Note the presence of EMD apparently closely associated with LAMP-1.

8.3.3. Formation of EMD, Fraction C and Fraction A precipitates

Commercially available lyophilized EMD has previously been shown to be soluble only in acidic solution (0.1% acetic acid) and to precipitate at neutral pH (Gestrelius et al., 1997). In the present study, although EMD, Fraction C and Fraction A also appeared to be readily soluble in 0.1% acetic acid (pH 4.1), based on the clear appearance of this solution, both EMD and Fraction A formed globular precipitates when added to GM (pH 7.4) and incubated on tissue culture plastic at 37°C for 24 h (in the absence of any cells), as shown by phase contrast microscopy and SEM (Fig. 8.9). In contrast, the low molecular weight Fraction C did not appear to form any type of precipitate, based on the absence of particulate material on the plastic surface (Fig. 8.9). When added to cultures of PDL cells for 24 h at 37°C, EMD and Fraction A were also found to precipitate and form spherical, aggregate-like structures (at the pH of GM (7.4)) that appeared to be closely associated with the cell surface (Fig. 8.10). However, no particulate material was detected when the PDL cells were cultured with Fraction C (Fig. 8.10). Moreover, Figure 8.11 show that the tissue culture plastic on which PDL cells were seeded and treated with EMD and Fraction A in GM at 37°C for 24 h exhibited precipitated globular aggregate-like structures on both the cell surface as well as on the tissue culture plastic surface, which could not be observed in higher magnification images in Figures 8.9 and 8.10. These results thus demonstrate that EMD and Fraction A precipitate on the PDL cell surface as well as on the plastic surface after 24 h of incubation in GM. In contrast, even in the presence of cells Fraction C produced no visible evidence of precipitation.

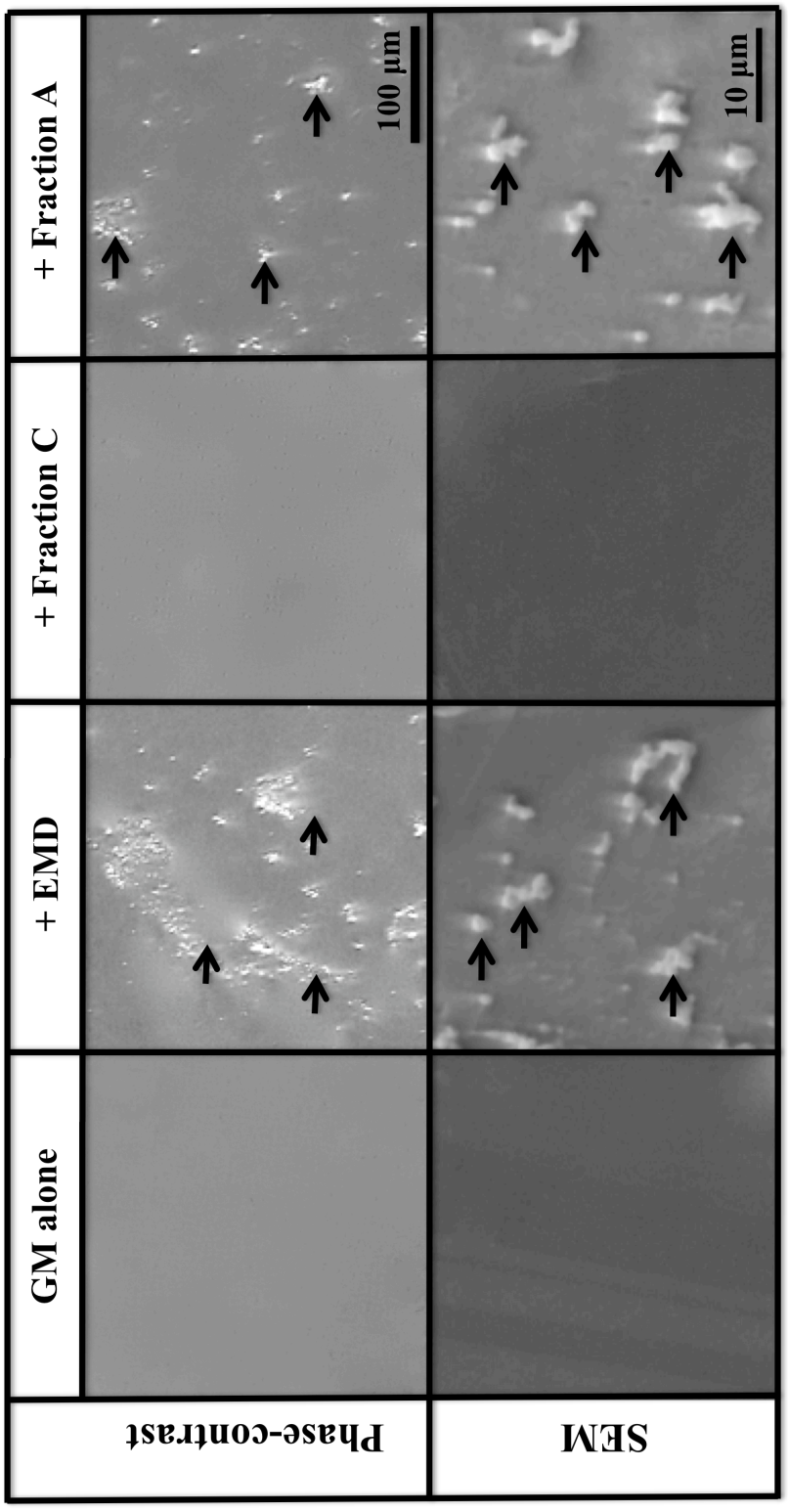


Figure 8.9. Phase-contrast and SEM micrographs of EMD, Fraction C and Fraction A (30 µg/ml) placed into GM and incubated at 37°C for 24 h in the absence of cells. Black arrows indicate the apparent precipitation of globular structures formed by EMD and Fraction A.

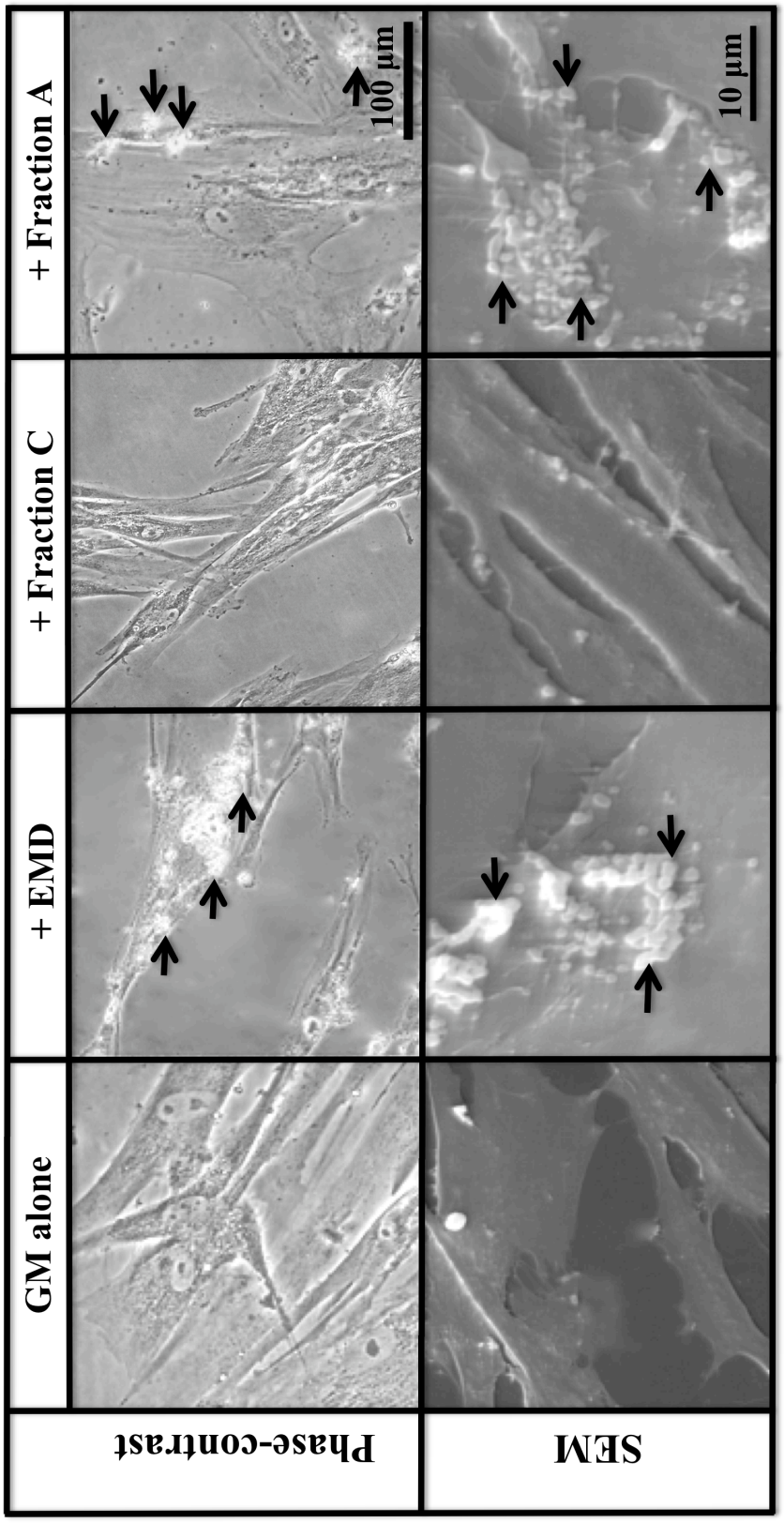


Figure 8.10. Phase-contrast and SEM micrographs of PDL cells cultured in GM in the absence and presence of EMD, Fraction C and Fraction A (30 µg/ml) at 37°C. Black arrows indicate globular-like structures on the PDL cell surface after 24 h. Note the absence of globular-like structures in cultures treated with Fraction C.

Figure 8.11. SEM micrographs of PDL cells cultured in GM alone, GM + EMD, GM + Fraction C and GM + Fraction A (all 30 µg/ml) at 37°C for 24 h. Black arrows indicate precipitation of EMD and Fraction A on the PDL cell surface after 24 h. White arrows indicate globular-like structures of EMD and Fraction A directly on the tissue culture plastic after 24 h. Note the absence of globular-like structures in the cultures in GM alone and with Fraction C.

8.4. Discussion

The PDL has previously been reported to contain a progenitor/stem cell-like population capable of multi-lineage differentiation and possibly playing a pivotal role in periodontal repair and regeneration (Amin et al., 2011; Singhatanadgit et al., 2009, Xu et al., 2009; Fujii et al., 2008; Chen et al., 2006). A number of biological mediators, including growth factors (Wnt, PDGF, BMP-2, IGF and TGF-β) and hormones (parathyroid hormone and dexamethasone), have been shown to elicit profound effects on PDL cell proliferation and osteogenic differentiation by acting as ligands that bind to the extracellular domain of transmembrane receptors. The subsequent changes in conformation/activity of the intracellular domains of the receptors (Kao et al., 2009; Zhao et al., 2002; Strayhorn et al., 1999; Chien et al., 1999; Takayama et al., 1997) lead to a cascade of protein kinase phosphorylation, ultimately resulting in the activation of transcription factors (e.g. Runx2) that bind to specific gene promoter sequences that regulate mRNA and, ultimately, protein production (Trippel et al., 1996; Barnes et al., 1999). Receptor-mediated endocytosis of ligand-receptor complexes is general considered as one of the mechanisms by which the level of cell surface receptors responsible for signal transduction is regulated (French et al., 1995; Seto et al., 2002). For example, TGFβ has been shown to bind to TGFβR-I and II and to transduce intracellular signals and the ligand-receptor complexes subsequently internalized via clatherin-mediated endocytosis (Anders et al., 1997). The ligands are dissociated from the complexes in the early endosomes and the receptors recycled back to the cell surface where they once again become available for further TGFβ-mediated signal transduction activity (Anders et al., 1997).

EMP has been reported to induce PDL cell attachment, proliferation and multi-lineage differentiation (osteogenesis, chondrogenesis and angiogenesis) *in vitro* and periodontal tissue regeneration *in vivo* (Gestrelius et al., 1997; Van der Pauw et al. 2000; Narukawa et al., 2007; Schlueter et al., 2007). Although it is not yet clear whether and which components of commercially prepared (heat-treated) porcine EMD bind to specific transmembrane receptor(s) and potentiate PDL cell proliferation and differentiation via signal transduction mechanism. However, previous reports examining the role of mouse amelogenins in tooth

development demonstrated that the full-length mouse recombinant amelogenin (M180) and its isoforms (M50) and (M73) bind to the receptor CD107a (also known as LAMP-1) and to the receptor CD63 (also known as LAMP-3), respectively, on mouse embryonic dental follicle cell membranes and transduce signals for odontogenic differentiation (Zhang et al., 2010; Tompkins et al., 2006; Iacob et al., 2008), indicating that certain component(s) within EMD may have an important signalling role in the process of cell differentiation. In addition, it was reported previously that when human osteoblasts were incubated with a crude preparation of EMP at 37°C for 3 h, protein component(s) were found to be internalized into cells via clathrin-coated pits, identified by simultaneous immunolabelling using anti-EMP and anti-AP-2, a clathrin adaptor protein which is responsible for linking the ligand-receptor complex (cargo) into the clathrin-coated pits intracellularly (Reseland et al., 2006). However, in this study the binding of EMP to the cell membrane and intracellular transport and fate of EMP were not examined (Reseland et al., 2006). Moreover, EMP is a complex mixture of various proteins including amelogenin, sheathlins and enamelin, and growth factors have also been found to be present in non-heat-treated crude preparations (Brookes et al., 1995; Hu et al., 1997). There is therefore uncertainty about the specificity of the antibody raised against crude EMP and thus the specific component(s) which may have been internalized into the osteoblasts (Reseland et al., 2006). In the present study it has been shown, for the first time, that at 4°C at least some component(s) of the commercial preparation of EMD and EMD components (Fraction C and Fraction A, isolated using industrial protein fractionation methodologies) bind to the PDL cell membrane and at 37°C these proteins are internalized and transported to the peri-nuclear region of the cells after 45 min, co-localizing with the TGN marker 58K. Moreover, after 3 h these protein components were found to have diffuse cytosolic distribution and were co-localized with LAMP-1-positive sub cellular lysosome-like structures. Similar to the present observations, a previous study using a recombinant mouse amelogenin isoform (M50) showed that this protein binds to the murine ameloblast (LS8) membrane after incubation at 4°C for 1 h and internalizes and co-localizes with LAMP-1-positive lysosomes when cultures were further incubated at 37°C for 1 h (Iacob et al., 2008). In addition, using recombinant mouse amelogenin (M180) it was reported that this protein internalizes into mouse osteoblasts (MC3T3) and ameloblasts (LS8) when incubated at 37°C for 1 h, the internalized amelogenin having a diffuse cytosolic distribution and co-localizing with LAMP-1-positive intracellular vesicle-like structures (Shapiro et al., 2007). A previous study using Wnt3a, a developmental protein which has previously also been shown to be responsible for bone homeostasis and repair (Blitzer and Nusse, 2006; Port and Basler, 2010),

showed that this protein binds to Wnt receptor (Frizzle) on murine fibroblast (murine L cell line) membrane and the ligand internalizes into the cell via receptor-mediated endocytosis and is transported to the TGN and lysosomes, similar to the present observations using EMD and the EMD components and previously reported observations using recombinant mouse amelogenins, as noted above, indicating that at least some components of EMD, Fraction C and Fraction A used here internalize via the process of receptor-mediated endocytosis (Iacob et al., 2008; Shapiro et al., 2007). It is also notable that, in the present study when the heterogeneous PDL cell population used here was treated with EMD, Fraction C and Fraction A, only a proportion of the cells were able to bind and internalize these proteins, suggesting that there is a subpopulation of cells within the PDL that expresses receptor(s) involved in direct interaction with EMD and the EMD components.

During porcine development EMP has been shown to be secreted by ameloblasts, specialized epithelial cells having unique extracellular projections mediating EMP secretion (tome's processes) (ten Cate et al., 1996; Heritier et al., 1982), and to precipitate on the surface of these same cells, promoting development of tooth-associated tissues (Deutsch et al., 1995). Similarly, commercially available Emdogain®/EMD has been shown to precipitate and form aggregate-like structures on the PDL cell surface *in vitro* and in the tissue microenvironment *ex vivo*, mimicking natural tooth-associated tissue development, noted above (Gestrelius et al., 1997). The present study observed that both EMD and the higher molecular weight Fraction of EMD (Fraction A) precipitated and formed globular aggregate-like structures on the PDL cell surface as well as on tissue culture plastic. Similarly, proline-rich hydrophobic full-length recombinant mouse amelogenin (M180) has been reported to also form insoluble supramolecular aggregates (2-3 million Daltons molecular size under physiological conditions) (Fincham et al., 1994), suggesting that the full-length amelogenin, one of the main components of EMD and Fraction A, might be responsible for the aggregate-like structures observed here. However, in contrast to the higher molecular weight components of EMD, it was observed here that the low molecular weight proteins (of Fraction C) did not precipitate and form such aggregate-like structures. Nevertheless, Fraction C, which contains mainly the 5 kDa peptide (TRAP) derived from full-length amelogenin, had the potential to stimulate chondrogenic, vasculogenic, angiogenic, neurogenic and gliogenic differentiation of PDL cells *in vitro* (Chapters 4-7), despite the lack of apparent ability to form precipitates and aggregates on the cell surface. It is thus likely that Fraction C acts by a receptor-mediated endocytosis mechanism rather than by a precipitation-related process.

However, the role of phagocytosis in EMD mode-of-action can not be entirely excluded since fibroblastic cells, which have been reported to be present in the PDL population (Kuru et al., 1998), have also been shown to be capable of internalizing insoluble macromolecules via phagocytosis (Groves et al., 2008; Lee et al., 1996). For example, collagen (300 kDa) is internalized by human gingival and PDL fibroblasts via 'phagosomes', a specialized intracellular phagocytic vesicle for intracellular breakdown (Lee et al., 1996); Beertsen et al., 1987). Although the present study did not investigate phagocytosis in relation to the uptake of EMD and Fraction A, it is possible that insoluble aggregates of these components could be subjected to phagocytic uptake and thereby generates some functional consequences.

In conclusion, the present study has shown that at least some components in Fraction A and the TRAP peptide in Fraction C can bind and be internalized via receptor-mediated endocytosis, and that putative receptor(s) specific for Fraction A and Fraction C are expressed by only a sub-population of PDL cells.

Chapter 9

9.1. General discussion

9.1.1. PDL cell plasticity

Stem cell-like populations within adult tissue are considered to play a key role in tissue homeostasis and the replenishment of cells that have died because of injury or disease (Weissman et al., 2000). Such 'stem' cells have now been identified and isolated from many tissues, including bone marrow (Jiang et al., 2002; Haynesworth et al., 1992), CNS (Johe et al., 1996), olfactory epithelium (Barnett et al., 2004), dental pulp (Gronthos et al., 2000), epidermis of the skin (Niemann et al., 2002; Cotsarelis et al., 1990), gastrointestinal track (Potten et al., 1998), BV (Asahara et al., 1997), skeletal muscle (Collins et al., 2005), cornea (Chen et al., 2004), heart (Laugwitz et al., 2005), adipose tissue (Zuk et al., 2001) and lung (Wu et al., 2004). A stem cell-like population has also been reported in the PDL by McCulloch (1987), who identified a population of cells exhibiting cytological features of stem cells, including small size, responsiveness to stimulatory growth factors and biological mediators, slow cell cycle time, higher number of population doublings, colony forming capacity and multi-lineage differentiation ability (osteogenic and adipogenic) *in vitro* (McCulloch et al., 1987).

A number of other studies have since reported the presence of a stem cell-like population within the PDL using a range of phenotypic markers such as STRO-1, CD29, CD44, CD105, CD106 and CD146 (Tomokiyo et al., 2008; Xu et al., 2009; Cheng et al., 2009). However, since these surface antigens are also expressed by several other types of adult human cells which are not considered stem cells, such as mature keratinocytes, T-lymphocytes, fibroblasts and glycophorin-A-positive red blood cells, the above phenotypic criteria may not be specific for identifying a PDL progenitor/stem cell-like population (Gronthos et al., 2003; Simmons and Torok-storb, 1991; Mareddy et al., 2007; Staquet et al., 1989; Weninger et al., 2000; Pickl et al., 1997). PDL tissue is highly vascular and also has dense sensory nerve innervations which indicate that precursors of endothelial and neural lineage might also be present in this tissue. However, the above studies examining the differentiation potential of PDL cells have been limited to mesenchymal pathways (osteogenic, adipogenic and chondrogenic) and detailed studies of the possible non-

mesenchymal differentiation pathways of PDL cells (e.g., vasculogenic, angiogenic, neurogenic and gliogenic) have previously not been carried out.

The present study cultured heterogeneous PDL cells firstly under non-differentiation inducing growth conditions and observed that these cells expressed mRNA transcripts of the early Runx2, PPARγ2, Sox-9, NGF and Notch1 genes associated with osteoblasts, adipocytes, chondrocytes, nerve and glial cells, respectively. Furthermore, when the PDL cells were cultured in lineage-specific differentiation media, they were able to undergo osteogenic, adipogenic, chondrogenic, vasculogenic, angiogenic, neurogenic and gliogenic differentiation *in vitro*, based on expression analysis of lineage-associated early and late genes and histological/immunocytochemical characteristics (Chapter 3), suggesting that the PDL may contain precursors capable of mesenchymal as well as non-mesenchymal lineage differentiation. Moreover, despite of lack of expression of the endothelial precursor markers VE-cadherin and CD31 by PDL cells when cultured under growth conditions (Xu et al., 2009; Zhou et al., 2008; Chapter 3), the cells cultured under endothelial media/differentiation conditions nevertheless formed endothelial-like cells *in vitro*, indicating that the PDL may contain a primitive cell population with the ability to differentiate into endothelial cells.

Although PDL cells were found to express mRNA transcripts of range of mesenchymal and non-mesenchymal pathway-associated genes under growth conditions, as noted above, only a certain proportion of cells within this heterogeneous cell population were found to undergo lineage-specific differentiation and form lineage-associated cells under differentiation conditions (between 7 to 36%; Chapter 3) *in vitro.* This suggests the possibility that the PDL may contain subpopulations of lineage-specific precursor cells. Singhatanadgit (2009) obtained direct evidence of 4 clonal cell subpopulations within the PDL which, although they expressed a similar phenotype (positive for CD29 and CD44 and negative for CD34 and CD45) and exhibited spindle-shaped morphology, nevertheless differed in their ability to proliferate and differentiate into osteogenic, adipogenic and chondrogenic lineages. Thus, while 1 of 4 cell clones (Clone 7) exhibited a prolonged total population doubling (60 population doublings before cell senescence) and the ability to differentiate into multiple lineages (osteoblasts, adipocytes and chondrocytes), the other 2 clones (Clones 5 and 6) exhibited only a limited population doubling level (20 population doublings before cell senescence) and differentiation potential (only osteogenic differentiation). One of the clonal populations (Clone 8) exhibited less than 20 population doublings before cell senescence and did not differentiate into any of the lineages noted above. These observations are thus

consistent with the presence of subpopulations of precursors within the PDL having varying differentiation potential. Furthermore, Xu (2009) showed that the PDL contains a STRO-1-positive cell population which exhibits the ability to proliferate extensively and differentiate into bone, fat and cartilage cells *in vitro,* in contrast to the observations reported by Singhatanadgit (2009) in which the heterogeneous PDL cell population was shown to be negative for STRO-1, including clone 7. These findings again indicate that the PDL is likely to contain a number of cell subpopulations with different proliferation, differentiation and phenotypic characteristics.

Fibroblast-like cells have been shown to be the predominant resident cell type of the PDL and such cells are mainly responsible for overall production and turnover of ECM. However, previous reports have suggested that the fibroblast population within the PDL is not homogeneous and may consist of different subpopulations with unique phenotypes and distinct functional activities such as osteogenic differentiation *in vitro* and bone formation *in vivo* (Hakkinen and Larjava, 1992; Irwin et al., 1994; Kuru et al., 1998). For example, Kuru (1998) showed that the PDL contains fibroblast subpopulations expressing high and low fibronectin, an extracellular matrix glycoprotein that binds to integrin receptors and regulates cell adhesion, migration, differentiation and wound healing (Pankov et al., 2002; Grinnell et al., 1984), despite similarities in size, granularity and morphology, again suggesting heterogeneity in PDL cell subpopulations. In addition to the possible presence of a range of lineage-associated precursors that give rise to multiple cell types, as evident by the proportion of cells which were found to be able to differentiate into mesenchymal and non-mesenchymal lineages as shown here, it is also possible that the PDL may also contain a subpopulation of 'primitive' stem cells, as discussed in **Section 9.1.3**.

9.1.2. Identification of bioactive components of EMD

Periodontitis is an infectious disease that results in a progressive loss of PDL, PDL-associated nerves, BV, cementum and AB and may ultimately lead to tooth loss. The goal of periodontal therapy is to restore the complex structure and function of the lost periodontal tissue. EMD (Institut Straumann, Basel, Switzerland), a commercially available heat-treated preparation of EMP extracted from developing pig, has been widely used in attempting to rebuild periodontal tissues (Cochran et al., 1999; Fincham et al., 1993; Margolis et al., 2006; Kanazashi et al., 2006; Donos et al., 2003; Sculean et al., 1999; Rathe et al., 2008), although clinical outcomes following EMD treatment have not always been consistent or predictable. For example, a histological study examining the effects of EMD on periodontal regeneration in a buccal dehiscence defect in humans observed that EMD-treated test sites exhibited a regenerated PDL with functionally oriented collagen fibres, cementum and AB compared with an untreated control group where little if any regeneration was observed (Heijl et al., 1997). In contrast, another study observed that only 2 out of 7 intrabony defects treated with EMD developed fully regenerated PDL, cementum and AB whereas the other 5 defects were characterized by insufficient bone regeneration (Sculean et al., 1999). Similarly, another clinical study observed that out of 10 intrabony defects only 3 exhibited full periodontal regeneration (Yukna and Mellonig, 2000). Similar discrepancies have also been reported in a number of animal studies, for example, Donos (2003) treated furcation degree III defects with EMD and observed that only 1 of 3 monkeys exhibited full closure with fully regenerated PDL, cementum and AB, the other 2 defects showing only partial closure with incomplete bone regeneration. Critical-size calvarial defects in rats treated with EMD have also been reported to exhibit little if any regenerated bone (Donos et al., 2004). In addition, while a number of *in vitro* studies have demonstrated that commercial preparations of EMD and crude preparations of EMP both stimulate bone-associated markers and bone-like nodule formation of PDL and other bone-forming cells (Gestrelius et al., 1997; Van der pauw et al. 2000; Nagano et al. 2004), other studies have shown that these preparations inhibit the expression of osteogenic genes and mineralized bone-like nodule formation *in vitro* (Hamma et al., 2008; Hakki et al., 2001).

The reason(s) for these discrepancies is not yet known, but it is possible that the components of EMP (including growth factors) vary qualitatively and quantitatively during different stages of enamel formation and crude extracts of EMP may therefore also vary in their components. EMP is synthesized by specialized epithelial cells (ameloblasts) during

tooth-supporting tissue and enamel development. The secretion of EMP can be observed during three distinct stages of enamel formation: The pre-secretory stage, the secretory stage and the maturation stage (Deutsch et al., 1995; ten Cate et al., 1996; Heritier et al., 1982). Although the major biosynthesis and secretion of EMP containing amelogenin, sheathlins, enamelin and tuftelin has been shown to take place in the secretory stage, the full-length amelogenin and its splicing forms (e.g., LRAP) are also secreted in the early pre-secretory and late maturation stages (Deutsch et al., 1995). Within hours after secretion, progressive proteolytic clipping of amelogenins have been shown to give rise to amelogenin peptides (including TRAP), and secreted peptides are gradually degraded and removed from the site by enzymatic digestion (Deutsch et al., 1995; Deutsch, 1989). Thus, the production and secretion of EMP components varies between different stages of enamel development.

In contrast to EMP, commercially available EMD is a heat-treated acid extract of EMP derived from six-month-old piglets (Heijl, 1997; Gestrelius et al., 1997), and the contents/components of EMD are rigidly controlled qualitatively and quantitatively (FDA approved quality control processes; Institut Straumann, Basel, Switzerland) to avoid variations between different batches (personal communications, Institut Straumann). Thus, different preparations of EMD are likely to be consistent qualitatively and quantitatively, suggesting the possibility that discrepancies in the effects of EMD on periodontal regeneration in humans, as noted above, may be at least partly due to varying experimental conditions, including age of patients, severity of periodontal and other systemic diseases (e.g., diabetes), life style (i.e., eating habits, smoking) and oral hygiene/removal of bacterial plaque prior to regenerative procedures (Armitage et al., 1999; Garrett, 1996). Similarly, discrepancies between animal studies could be due to the use of different animal models/defects, varying age and healing conditions.

Several attempts have been made to fractionate freshly isolated porcine EMP in order to delineate the active components in this heterogeneous protein mixture. For example, Iwata (2002) fractionated EMP from developing porcine teeth and reported that the fraction containing 20 kDa peptides exhibited osteoinductive properties, as assessed by stimulation of ALP activity and mineralized nodule formation and up-regulation of OC, BSP and ALP genes in the mouse bone marrow stromal cell line ST2. However, the methodology used in this study could not exclude the possibility that the osteoinductive fraction may have contained additional lower-molecular weight components which were bioactive. In addition, crude preparations of EMP are known to contain BMP and TGF β growth factors, which may have

also contributed to the osteoinductive effects observed in this report (Iwata et al., 2002). This is consistent with the conclusion reported by Nagano (2006), who showed that the osteoinductive property of EMP fraction consisting of proteins of size > 10 kDa (Fraction 3; Nagano et al., 2006) was due to the presence of the TGF β1 growth factor. These observations suggest that more purified protein components are needed in order to assess the potential value of EMP in PDL and bone regeneration.

The present study used two sub-fractions of the commercial preparation of EMD (Fraction C and Fraction A) that were obtained by industrial scale protein fractionation methodologies (Mumulidu et al., 2007), and examined the effects of these Fractions on bone, BV, nerve and glial cell formation by PDL cells *in vitro*. The results showed, for the first time, that the low (< 6 kDa; Fraction C) and the high (> 6 kDa; Fraction A) molecular weight Fractions of EMD differentially regulated these pathways of PDL cell differentiation. Thus, while Fraction C strongly suppressed bone-forming activity, Fraction C promoted BV, nerve and glial cell formation by PDL cells (Chapter 4, 6 and 7). In contrast, Fraction A induced bone-forming activity but strongly suppressed BV, nerve and glial cell differentiation (Chapters 4, 6 and 7). Since the amelogenin-derived TRAP is a main component of Fraction C and LRAP is an amelogenin-derived component of Fraction A (Mumulidu et al., 2007; Fincham et al., 1994), the present study also examined the effects of chemically synthesized TRAP and LRAP on osteogenic differentiation. The results showed that, as with Fraction C, TRAP suppressed the bone-forming activity of PDL cells whereas, as with Fraction A, LRAP induced osteogenic differentiation of the PDL cells *in vitro* (Chapter 4). Moreover, the TRAP peptide was also found to stimulate BV, nerve and glial cell formation, as found with Fraction C (Chapters 6-8), suggesting that LRAP and TRAP may be at least partly responsible for the regenerative activities of EMD.

By amino acid sequence comparison of TRAP, LRAP and amelogenin, the study also identified the TCT peptide as the unique 12-amino acid C-terminal sequence present in TRAP, and LCT as the unique 23-amino acid C-terminal sequence present in LRAP. TCT and LCT were therefore chemically synthesized and examined for their effects on PDL cell osteogenesis, the results showing, for the first time, that the TCT sequence suppressed osteogenic differentiation whereas LCT stimulated this process (Chapter 4). These data suggest that these sequences are the active domains of the parent TRAP and LRAP peptides, respectively. The mechanism(s) of action of these peptides is not yet known, but it is noteworthy that initial experiments using TRAP and LRAP showed that they differentially

regulate the expression of Wnt3a and Wnt5a developmental genes which have been shown to induce osteogenesis (Williams et al., 2009; Wu et al., 2004) of PDL cells when cultured in GM for 6 h (Appendix Material 4.1). Thus, TRAP was found to suppress both of these Wnt genes, whereas LRAP up-regulated Wnt3a and 5a, indicating that TRAP and LRAP may possibly regulate osteogenic differentiation via a Wnt-dependent signal transduction pathway.

9.1.3. Interaction of EMD components with PDL cells

It has been shown here that Fraction C and Fraction A differentially regulated a range of PDL cell differentiation activities, including osteogenesis, adipogenesis, chondrogenesis, vasculogenesis, angiogenesis, neurogenesis and gliogenesis *in vitro* (Chapters 4-7). Although the mechanism(s) by which these Fractions exert their biological activity is not yet known, it is likely to involve interaction of one or more components directly with the target PDL cells. Thus, the possible binding and internalization of EMD and the EMD Fractions into PDL cells was also examined here, the results indicating that at least some component(s) of Fraction C and Fraction A bind to the cell membrane and are internalized most probably via receptor-mediated endocytosis (Chapter 8). Although receptor(s) on the PDL cells responsible for the binding and uptake of these EMD components have not yet been identified, the CD63 and CD107a receptors on mouse dental follicle cells have previously been shown to be responsible for binding mouse full-length amelogenin (M180) and the amelogenin (M50) isoform and for transducing signals for odontogenic differentiation, as well as for internalization of these mouse amelogenins (Zhang et al., 2010; Tompkins et al., 2006; Iacob et al., 2008). These findings suggest the possibility that the amelogenin-derived components studies here may regulate PDL cell differentiation via signal transduction mechanism(s) and become internalized via receptor mediated endocytosis.

It was observed here that only a certain proportion of PDL cells were able to internalize Fraction C and Fraction A. Thus, the results in the present study showed that only approximately 6 and 12% of the cells within heterogeneous PDL cell population were able to internalize Fraction C and Fraction A, respectively, when cultured under growth conditions (Chapter 8). These results thus indicate that there may be subpopulation of cells within the PDL expressing receptor(s) responsible for binding and uptake of Fraction C and Fraction A and capable of specifically responding to Fraction C/TRAP and Fraction A. Similarly, it was also observed here that optimal concentrations of Fraction C and TRAP stimulated only limited proportion of the PDL cell population to form endothelial, nerve and glial cells in culture (Alizarin red staining, used to quantify the levels of bone-like nodule formation/mineralization by the PDL cultures, did not allow for measurement of the proportion of bone-forming cells, and it was not possible to determine the proportion of PDL cells capable of osteogenic differentiation in the presence of Fraction A). Thus, when PDL cells were cultured under differentiation conditions (endothelial, neural and glial media) in the presence of Fraction C and TRAP for up to 5 weeks, 15-30% of the cells exhibited

characteristics of endothelial, nerve and glial cells *in vitro*, the remaining cells exhibiting a spindle-shape fibroblastic morphology (Chapter 6 and 7), again suggesting that there may be a Fraction C/TRAP-specific subpopulation of cells within the PDL. It is notable that the proportion of cells with the ability to internalize Fraction C was found to be markedly lower than the proportion of cells that developed endothelial, nerve and glial characteristics in the presence of Fraction C/TRAP. The reason for this discrepancy is not yet clear, but it is possible to speculate that Fraction C/TRAP might have induced proliferation and expansion of a Fraction C/TRAP-responsive subset of cells which could subsequently be induced to undergo differentiation to lineage-specific cells under differentiation-stimulating conditions *in vitro*. It is also possible that the relatively low proportion of Fraction C/TRAP-responsive cells may comprise the 'true' stem cells with the potential for multi-lineage specification, as described above (**Section 9.1.1**).

9.2. Concluding remarks

This thesis has demonstrated that the PDL contains one or more subpopulations of cells capable of differentiation to multiple lineages *in vitro*. Moreover, Fraction C and Fraction A derived from EMD were found to differentially regulate the apparent multi-lineage specification of the PDL cells, with Fraction C up-regulating chondrogenic, vasculogenic, angiogenic, neurogenic and gliogenic genes and 'terminal' differentiation and Fraction A stimulating osteogenic genes and terminal osteogenic differentiation. In addition, the TRAP and LRAP peptides of Fraction C and Fraction A, respectively, were found to be at least partly responsible for the activities of these two Fractions.

9.3. Future work

9.3.1. Effects of LRAP-depleted Fraction A and TRAP-depleted Fraction C on PDL cell differentiation

This thesis has provided evidence that higher molecular weight EMD Fraction A stimulates osteogenesis and lower molecular weight EMD Fration C stimulated chondrogenesis, vasculogenesis, angiogenesis, neurogenesis and gliogenesis. Moreover, chemically synthetic LRAP, major component of Fraction A, and TRAP, major component of Fraction C, exhibited similar bioactivities to naturally occuring heterogeneous Fractions. Thus, in order to examine whether the LRAP and TRAP peptides are solely responsible for the bioactivities of Fraction A and Fraction C, respecitvely, it may be useful to prepare Fraction A depleted of LRAP and Fraction C depleted of TRAP and determine the bioactivities of these LRAP and TRAP depleted Fractions on PDL cell differentiation. This will establish whether LRAP and TRAP alone are responsible for the bioactivity of whole EMD preparation and the EMD Fractions or there are other components present in EMD/Fractions which also potentially regulate the differentiation pathways investigated in this thesis.

9.3.2. Effect of the LCT and TCT peptides on vasculogenesis, angiogenesis, neurogenesis and gliogenesis of PDL cells

This thesis has provided evidence that the amelogenin-derived peptides LRAP and TRAP and their specific amino acid sequences LCT and TCT differentially regulate bone-forming cell activity *in vitro*. Thus, while LRAP and LCT have been shown to stimulate osteogenic differentiation of range of adult human bone-forming cells (PDL cells, AB cells, osteogenic PDL clone 7 and BMSC), TRAP and TCT suppressed this activity *in vitro*. In addition, the TRAP peptide was shown here, for the first time, to stimulate chondrogenic, vasculogenic, angiogenic, neurogenic and gliogenic differentiation *in vitro.* However, the effects of the newly-identified LCT and TCT sequences on these differentiation pathways have not hitherto been examined and experiments are now needed to examine the effects of these peptides on the *in vitro* assays used in this thesis for PDL cell chondrogenesis, vasculogenesis, angiogenesis, neurogenesis and gliogenesis. This will establish whether LCT and TCT are the specific amino acid sequences of the parent LRAP and TRAP peptides, and their potential efficacy for therapeutic applications in future.

9.3.3. Identification of the minimal amino acid sequences within LCT and TCT for inducing cell differentiation

Protein homology analysis of the TCT and LCT amino acid sequences using the National Centre for Biotechnology Information (NCBI) protein database and BLAST software (NCBI, Bethesda, MD) identified a number of amino acid sub-sequences that were most 'conserved' within TCT and LCT sequences among > 100 different animal species, as shown in Table 12. This indicates the possibility that these smaller unique C-terminal amino acid sequences may retain the bioactivity of the parent LRAP/LCT and TRAP/TCT and may have the potential to modulate the multiple lineages of putative precursor cells. Identifying such conserved minimal amino acid sequences, as noted in Table 12, would have a number of important advantages: (i) chemical synthesis would be cost effective and can be produced in a large quantity with high purity for clinical use; (ii) likely to have markedly decreased immunogenicity; (iii) chemical modifications of the peptides, to prevent break-down, target to specific cells/tissue and enhance activity, would be relatively easy.

Table 12. Amelogenin-derived peptide sequences

LRAP	MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHP SLLPDLPLEAWPATDKTKREEVD
TRAP	MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHP YTSYGYEPMGGW
LCT	SLLPDLPLEAWPATDKTKREEVD
LCT conserved 1	LPDLPLEAWPAT
LCT conserved 2	PDLPLEAWPAT
LCT conserved 3	PDLPLEAW
TCT	YTSYGYEPMGGW
TCT conserved 1	GYEPMGGW
TCT conserved 2	GYEPMG

$-NH_2$ → -COOH

9.3.4. Effects of LCT and TCT on other non-dental cells

In addition to PDL cells, determining the effects of the peptides may prove beneficial in increasing the spectrum of possible clinical use for regenerating non-dental adult tissues. For example, use of BMSC and primary chondrocitic cultures obtained from synovial joints could be used to examine the chondrogenic differentiation activity of the peptides. Vasculogenic differentiation, reported to take place mainly in developing embryos, female reproductive track/endometrium and healing wounds (Vailhë et al., 2001), could be studied using the human embryonic stem cell line (MEL-1; Millipore), primary cultures from endometrium and

developing/foetal heart. In addition, the TRAP peptide has been shown here to have the ability to stimulate neurogenesis of PDL cells and generate differentiated nerve-like cells, and it may therefore be useful to examine its effect on primary cultures isolated from sensory neuron compartments such as dorsal root ganglion, spinal cord and enteric tissue.

Further, since initial studies in this thesis demonstrated that TCT suppresses bone-forming cells, it might therefore be of value for blocking the development/progression of bone-associated tumours. The peptide could therefore be examined for its effects on the growth of osteosarcoma cell lines (e.g., MG63, Saos-2, HOS) and the expression of key cell cycle-associated genes involved in tumourigenesis (e.g. p53, INK4, COPS4 and CDKs and CDKLs).

9.3.5. Molecular mechanism(s) involved in TRAP/TCT and LRAP/LCT-mediated differentiation

The molecular mechanisms by which these peptides elicit their biological activities are not yet known, but in initial experiments it was found that TRAP suppressed both the Wnt 3a and 5a genes, developmental factors which have been shown to induce osteogenesis (Williams et al., 2009; Wu et al., 2004). In contrast, LRAP up-regulated the mRNA transcripts of both Wnt3a and 5a (Appendix Material 4.1), indicating that the differential regulation of osteogenic differentiation by TRAP and LRAP may be in part through signal transduction pathways associated with Wnt proteins. In addition, since a number of other Wnt ligands (i.e., Wnt-1, -2, -4, -7a, -7b) have previously been shown to regulate vasculogenic, angiogenic and neurogenic differentiation during embryonic development (Franco et al., 2009; Inestrosa et al., 2010), it is possible that the TRAP and TCT and LRAP and LCT peptides might differentially affect the expression of these other Wnt genes of PDL cells. Further, the functional activity of the peptides can be established by examining the effects of the peptides following si-RNA-mediated specific Wnt gene knockdown on terminal differentiation *in vitro*, which may be useful in identifying a possible molecular basis by which these peptides control lineage specification.

In addition to the specific Wnt gene pathways, cDNA PCR array (SABioscience, Crawly, UK) analysis of the changes in the expression of genes involved in a number of 'key' signal transduction pathways (e.g., FGF, Notch, Hedgehog, TGFβ, pluripotency maintenance pathways) associated with stem cell regulation may be useful in identifying other early molecular events involved in TRAP/TCT and LRAP/LCT-mediated PDL precursor/stem cell differentiation. Moreover, cDNA PCR arrays involving each differentiation pathway investigated in this thesis (i.e., osteogenic, adipogenic, chondrogenic, vasculogenic, angiogenic, neurogenic and gliogenic) may also be useful in delineating the pathway-specific mechanism(s) by which TRAP/TCT and LRAP/LCT differentially regulate the above differentiation pathways.

9.3.6. Identification and characterization of subsets of TRAP and LRAP-responsive cells and TRAP and LRAP-specific receptor(s)

Although the TRAP peptide was found to bind and internalize into the PDL cells, the binding and intracellular fate of LRAP and the TCT and LCT amino acid sequences derived from TRAP and LRAP have not yet been studied, nor whether these peptides are recognized by only a certain subset of the PDL cells, as indicated by the results obtained here using Fraction C and Fraction A. In addition, it was observed that only a certain proportion of cells exhibited endothelial, neural and glial cell-like characteristics when cultured in the presence of Fraction C/TRAP. It is therefore possible that a Fraction C/TRAP-specific subpopulation of cells may have the ability to differentiate into non-mesenchymal lineages and that a Fraction A/LRAP-specific subpopulation of cells may have mesenchymal lineage characteristics *in vitro*. Fraction C/TRAP- and Fraction A/LRAP-specific subpopulations within heterogeneous PDL cultures could be isolated by seeding PDL cells on Fraction C/TRAP and Fraction A/LRAP-pre-coated plates, washing away the non-attached cells and recovering the cells which had bound to the Fraction C/TRAP and Fraction A/LRAP-coated surfaces for further characterization (i.e., proliferation ability, multi-lineage differentiation potential and functional characteristics, FCM analysis for the expression of stem cell-associated markers such as Oct3/4, nanog, rex-1, STRO-1 and MUC18/CD146).

Identification of the TRAP/TCT and the LRAP/LCT-specific receptor(s) could help to delineate the signal transduction pathway(s) of these peptides. This could be carried out by electrophoretic analysis of specific TRAP/TCT and LRAP/LCT-binding surface protein(s) obtained from TRAP/TCT and LRAP/LCT-positive cells isolated by panning techniques, as described above. For example, the primary PDL cell population could be seeded on to biotinylated-TRAP/TCT and -LRAP/LCT-pre-coated tissue culture plates, cell population that bound specifically to the peptide-coated plates detached using EDTA and then streptavidin affinity used to isolate the specific receptor-biotinylated peptide complexes. Determining the receptor(s) involved in peptide binding and uptake might then be used for modulating the biological activity/efficacy of the peptides by intracellular trafficking drugs, by agents and by biological and chemical modification of the peptide-receptor interaction.

9.3.7. Evaluation of bone regenerative properties of LRAP and LCT *in vivo*

The critical-size calvarial defect model treated via Guided Bone Regeneration (GBR) (Bosch et al., 1998; Donos et al., 2004; Mardas et al., 2002; Mardas et al., 2008) can be used to investigate the ability of LRAP and LCT to promote intra-membranous bone regeneration. The critical-size calvarial defect is generally considered as an osseous defect that cannot heal on its own (Schmitz & Hollinger, 1986), but can sometimes be successfully treated via guided bone regeneration (Appendix Materials 4.2 and 4.3) (Dahlin et al., 1988, Donos et al., 2004; Mardas et al., 2002; Donos et al., 2011). This has been achieved via surgical placement of a cell-occlusive barrier membrane on the bone surface, preventing the invasion of non-bone forming cells from the surrounding soft tissues but enabling re-population of the defect site by osteoprogenitor cells originating from the skull diploe (spongy bone structure) (Hammerle et al., 1995; Donos et al., 2004; Retzepi *et al.*, 2007; Donos et al., 2011).

The absorbable collagen sponge (ACS) could be used as a selective carrier material to deliver the LRAP and LCT peptides before placement of the resorbable membrane, as previously used successfully with osteogenesis-inducing factors for enhancing bone healing (Donos et al., 2004; Retzepi *et al.*, 2007; Donos et al., 2011). A dose-effect of LRAP and LCT (e.g., from 0.1 to 1 μg/ml) would need to be established. Following healing periods between 7 to 30 days, control and test sites are removed and processed for qualitative and quantitative

histological evaluation of undecalcified sections, with new bone formation histomorphometrically measured. In replicate experiments animals could be sacrificed at 5 and 10 days post-operatively, the tissue within the osseous defect could be obtained for RNA extraction and evaluated using Affymetrix oligonoucletide microarrays (Rat GeneArray St1.0s) to examine the molecular changes induced by LRAP and LCT.

BIBLIOGRAPHY

Aguayo-Mazzucato C, Bonner-Weir S (2010). Stem cell therapy for type 1 diabetes mellitus. Nature Reviews Endocrinology 6(3):139-148.

Ahrens PB, Solursh M, Reiter RS (1977). Stage-related capacity for limb chondrogenesis in cell culture. Developmental Biology 60(1):69-82.

Alghisi GC, Ponsonnet L, R¸egg C (2009). The integrin antagonist cilengitide activates V 3, disrupts VE-cadherin localization at cell junctions and enhances permeability in endothelial cells. PloS one 4(2):e4449.

Alliston T, Choy L, Ducy P, Karsenty G, Derynck R (2001). TGF- -induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. The EMBO Journal 20(9):2254-2272.

Alonso L, Fuchs E (2003). Stem cells of the skin epithelium. Proceedings of the National Academy of Sciences of the United States of America 100(Suppl 1):11830.

Ambrosi D, Bussolino F, Preziosi L (2005). A review of vasculogenesis models. Computational and Mathematical Methods in Medicine 6(1):1-19.

Amin HD, Olsen I, Knowles J, Donos N (2011). A procedure for identifying stem cell compartments with multi-lineage differentiation potential. Analyst 136(7):1440-1449.

Anderson HC (1989). Mechanism of mineral formation in bone. Laboratory investigation 60(3):320-330.

Anderson HC (2003). Matrix vesicles and calcification. Current rheumatology reports 5(3):222-226.

Anderson RGW (1998). The caveolae membrane system. Annual review of biochemistry 67(1):199-225.

Anderson RGW, Brown MS, Goldstein JL (1977). Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell 10(3):351-364.

Angelastro JM, Klimaschewski L, Tang S, Vitolo OV, Weissman TA, Donlin LT, et al. (2000). Identification of diverse nerve growth factor-regulated genes by serial analysis of gene expression (SAGE) profiling. Proceedings of the National Academy of Sciences of the United States of America 97(19):10424.

Araujo MG, Lindhe J (1998). GTR treatment of degree III furcation defects following application of enamel matrix proteins. Journal of Clinical Periodontology 25(6):524-530.

Armitage GC (1999). Development of a classification system for periodontal diseases and conditions. Annals of Periodontology 4(1):1-6.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science 275(5302):964-966.

Atsumi Y, Matsumoto K, Sakuda M, Maeda T, Kurisu K, Wakisaka S (1999). Altered distribution of Schwann cells in the periodontal ligament of the rat incisor following resection of the inferior alveolar nerve: an immunohistochemical study on S-100 proteins. Brain research 849(1-2):187-195.

Aubin JE (1998). Bone stem cells. Journal of Cellular Biochemistry 72(S30-31):73-82.

Aubin JE (2001). Regulation of osteoblast formation and function. Reviews in endocrine & metabolic disorders 2(1):81-94.

Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N (2003). Angiogenesis assays: a critical overview. Clinical chemistry 49(1):32-40.

Baksh D, Boland GM, Tuan RS (2007). Cross talk between Wnt signaling pathways in human mesenchymal stem cells leads to functional antagonism during osteogenic differentiation. Journal of cellular biochemistry 101(5):1109-1124.

Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA (1999). Growth factor regulation of fracture repair. Journal of Bone and Mineral Research 14(11):1805-1815.

Barnett SC, Chang L (2004). Olfactory ensheathing cells and CNS repair: going solo or in need of a friend? TRENDS in Neurosciences 27(1):54-60.

Bashour AM, Bloom GS (1998). 58K, a microtubule-binding Golgi protein, is a formiminotransferase cyclodeaminase. Journal of Biological Chemistry 273(31):19612-19617.

Bauer SM, Bauer RJ, Velazquez OC (2005). Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. Vascular and endovascular surgery 39(4):293.

Beertsen W (1987). Collagen phagocytosis by fibroblasts in the periodontal ligament of the mouse molar during the initial phase of hypofunction. Journal of Dental Research 66(12):1708-1712.

Beertsen W, McCulloch CAG, Sodek J (1997). The periodontal ligament: a unique, multifunctional connective tissue. Periodontology 2000 13(1):20-40.

Bertl K, An N, Bruckmann C, Dard M, Andrukhov O, Matejka M, et al. (2009). Effects of Enamel Matrix Derivative on Proliferation/Viability, Migration, and Expression of Angiogenic Factor and Adhesion Molecules in Endothelial Cells In Vitro. Journal of periodontology 80(10):1622-1630.

Bhatheja K, Field J (2006). Schwann cells: origins and role in axonal maintenance and regeneration. The International Journal of Biochemistry & Cell Biology 38(12):1995-1999.

Birchmeier C, Nave KA (2008). Neuregulin 1, a key axonal signal that drives Schwann cell growth and differentiation. Glia 56(14):1491-1497.

Blanpain C, Fuchs E (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. Nature Reviews Molecular Cell Biology 10(3):207-217.

Blitzer JT, Nusse R (2006). A critical role for endocytosis in Wnt signaling. BMC Cell Biology 7(1):28.

Blum B, Bar-Nur O, Golan-Lev T, Benvenisty N (2009). The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells. Nature biotechnology 27(3):281-287.

Blum B, Benvenisty N (2008). The tumorigenicity of human embryonic stem cells. Advances in Cancer research 100(133-158.

Bodur A, Bodur H, Bal B, Balos K (2001). Generalized aggressive periodontitis in a prepubertal patient: A case report. Quintessence Int 32(4):303-308.

Bonta H, Llambes F, Moretti AJ, Mathur H, Bouwsma OJ (2003). The use of enamel matrix protein in the treatment of localized aggressive periodontitis: A case report. Quintessence international 34(4):247-252.

Borges J, Tegtmeier FT, Padron NT, Mueller MC, Lang EM, Stark GB (2003). Chorioallantoic membrane angiogenesis model for tissue engineering: a new twist on a classic model. Tissue Engineering 9(3):441-450.

Bosch C, Melsen B, Vargervik K (1998). Importance of the critical-size bone defect in testing bone-regenerating materials. Journal of Craniofacial Surgery 9(4):310.

Boyan BD, Weesner TC, Lohmann CH, Andreacchio D, Carnes DL, Dean DD, et al. (2000). Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. Journal of periodontology 71(8):1278-1286.

Brennan TJ, Edmondson DG, Olson EN (1990). Aberrant regulation of MyoD1 contributes to the partially defective myogenic phenotype of BC3H1 cells. The Journal of cell biology 110(4):929-937.

Brett PM, Parkar M, Olsen I, Tonetti M (2002). Expression profiling of periodontal ligament cells stimulated with enamel matrix proteins in vitro: a model for tissue regeneration. Journal of dental research 81(11):776-780.

Brey EM, Uriel S, Greisler HP, McIntire LV (2005). Therapeutic neovascularization: contributions from bioengineering. Tissue Engineering 11(3-4):567-584.

Brookes SJ, Robinson C, Kirkham J, Bonass WA (1995). Biochemistry and molecular biology of amelogenin proteins of developing dental enamel. Archives of Oral Biology 40(1):1-14.

Bruno B, Rotta M, Patriarca F, Mordini N, Allione B, Carnevale-Schianca F, et al. (2007). A comparison of allografting with autografting for newly diagnosed myeloma. New England Journal of Medicine 356(11):1110-1120.

Buchmann R, Nunn ME, Dyke TEV, Lange DE (2002). Aggressive periodontitis: 5-year follow-up of treatment. Journal of periodontology 73(6):675-683.

Byers MR (2004). Sensory innervation of periodontal ligament of rat molars consists of unencapsulated Ruffini-like mechanoreceptors and free nerve endings. The Journal of Comparative Neurology 231(4):500-518.

Byers MR, Taylor PE, Khayat BG, Kimberly CL (1990). Effects of injury and inflammation on pulpal and periapical nerves*. Journal of Endodontics 16(2):78-84.

Cancino J, Torrealba C, Soza A, Yuseff MI, Gravotta D, Henklein P, et al. (2007). Antibody to AP1B adaptor blocks biosynthetic and recycling routes of basolateral proteins at recycling endosomes. Molecular biology of the cell 18(12):4872-4884.

Caton J, Nyman S (1980). Histometric evaluation of periodontal surgery I. The modified Widman flap procedure. Journal of Clinical Periodontology 7(3):212-223.

Cattaneo V, Rota C, Silvestri M, Piacentini C, Forlino A, Gallanti A, et al. (2003). Effect of enamel matrix derivative on human periodontal fibroblasts: proliferation, morphology and root surface colonization. An in vitro study. Journal of Periodontal Research 38(6):568-574.

Cerny R, Slaby I, Hammarstr̂m L, Wurtz T (1996). A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. Journal of Bone and Mineral Research 11(7):883-891.

Chang HY, Sneddon JB, Alizadeh AA, Sood R, West RB, Montgomery K, et al. (2004). Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS biology 2(2):e7.

Chapman SC, Collignon J, Schoenwolf GC, Lumsden A (2001). Improved method for chick whole embryo culture using a filter paper carrier. Developmental Dynamics 220(3):284-289.

Chen D, Zhao M, Mundy GR (2004). Bone morphogenetic proteins. Growth factors 22(4):233-241.

Chen JW, Murphy TL, Willingham MC, Pastan I, August JT (1985). Identification of two lysosomal membrane glycoproteins. The Journal of cell biology 101(1):85-95.

Chen SC, Marino V, Gronthos S, Bartold PM (2006). Location of putative stem cells in human periodontal ligament. Journal of Periodontal Research 41(6):547-553.

Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, et al. (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell 133(6):1106-1117.

Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ (2004). Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 22(3):355-366.

Cheng MT, Yang HW, Chen TH, Lee OKS (2009). Isolation and characterization of multipotent stem cells from human cruciate ligaments. Cell Proliferation 42(4):448-460.

Chi GF, Kim M, Kim DW, Jiang MH, Son Y (2010). Schwann cells differentiated from spheroid-forming cells of rat subcutaneous fat tissue myelinate axons in the spinal cord injury. Experimental Neurology.

Chi GF, Kim M, Kim DW, Jiang MH, Son Y (2010). Schwann cells differentiated from spheroid-forming cells of rat subcutaneous fat tissue myelinate axons in the spinal cord injury. Experimental Neurology 222(2):304-317.

Cho MI, Garant PR (2000). Development and general structure of the periodontium. Periodontology 2000 24(1):9-27.

Chung U, Kawaguchi H, Takato T, Nakamura K (2004). Distinct osteogenic mechanisms of bones of distinct origins. Journal of Orthopaedic Science 9(4):410-414.

Citores L, Khnykin D, S¯rensen V, Wesche J, Klingenberg O, Wied ocha A, et al. (2001). Modulation of intracellular transport of acidic fibroblast growth factor by mutations in the cytoplasmic receptor domain. Journal of cell science 114(Pt 9):1677-1689.

Cochran DL, Wozney JM (1999). Biological mediators for periodontal regeneration. Periodontology 2000 19(40-58.

Cockerill GW, Gamble JR, Vadas MA (1995). Angiogenesis: models and modulators. International review of cytology 159(113-160.

Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, et al. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122(2):289-301.

Conner SD, Schmid SL (2003). Regulated portals of entry into the cell. Nature 422(6927):37-44.

Cortellini P, Carnevale G, Sanz M, Tonetti MS (1998). Treatment of deep and shallow intrabony defects A multicenter randomized controlled clinical trial. Journal of Clinical Periodontology 25(12):981-987.

Cotsarelis G, Sun TT, Lavker RM (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61(7):1329-1337.

Coura GS, Garcez RC, de Aguiar C, Alvarez-Silva M, Magini RS, Trentin AG (2008). Human periodontal ligament: a niche of neural crest stem cells. Journal of periodontal research 43(5):531-536.

Czasch S, Paul S, Baumg‰rtner W (2006). A comparison of immunohistochemical and silver staining methods for the detection of diffuse plaques in the aged canine brain. Neurobiology of aging 27(2):293-305.

D'Amore PA, Thompson RW (1987). Mechanisms of angiogenesis. Annual review of physiology 49(1):453-464.

DaCosta Byfield S, Major C, Laping NJ, Roberts AB (2004). SB-505124 is a selective inhibitor of transforming growth factor- type I receptors ALK4, ALK5, and ALK7. Molecular pharmacology 65(3):744-752.

Dahlin C, Linde A, Gottlow J, Nyman S (1988). Healing of bone defects by guided tissue regeneration. Plastic and Reconstructive Surgery 81(5):672-676.

Dean DD, Lohmann CH, Sylvia VL, Cochran DL, Liu Y, Boyan BD, et al. (2000). Effect of porcine fetal enamel matrix derivative on chondrocyte proliferation, differentiation, and local factor production is dependent on cell maturation state. Cells Tissues Organs 171(2-3):117-127.

Demir R, Seval Y, Huppertz B (2007). Vasculogenesis and angiogenesis in the early human placenta. Acta Histochemica 109(4):257-265.

Deutsch D, Catalano-Sherman J, Dafni L, David S, Palmon A (1995). Enamel matrix proteins and ameloblast biology. Connective tissue research 32(1):97-107.

Deutsch D, Palmon A, Fisher LW, Kolodny N, Termine JD, Young MF (1991). Sequencing of bovine enamelin (" tuftelin") a novel acidic enamel protein. Journal of Biological Chemistry 266(24):16021.

DiBattista P, Bissada NF, Ricchetti PA (1995). Comparative effectiveness of various regenerative modalities for the treatment of localized juvenile periodontitis. Journal of periodontology 66(8):673-678.

Donato R, Miljan EA, Hines SJ, Aouabdi S, Pollock K, Patel S, et al. (2007). Differential development of neuronal physiological responsiveness in two human neural stem cell lines. BMC neuroscience 8(1):36.

Dong ZW, Cheng YZ, Zhao J, Su L, Zhao BX, Zhang Y, et al. (2010). Discovery of a benzoxazine derivative promoting angiogenesis in vitro and in vivo. Journal of cellular physiology 223(1):202-208.

Donos N, Bosshardt D, Lang N, Graziani F, Tonetti M, Karring T, et al. (2004). Bone formation by enamel matrix proteins and xenografts: an experimental study in the rat ramus. Clinical Oral Implants Research 16(2):140-146.

Donos N, Bosshardt D, Lang N, Graziani F, Tonetti M, Karring T, et al. (2005). Bone formation by enamel matrix proteins and xenografts: an experimental study in the rat ramus. Clinical Oral Implants Research 16(2):140-146.

Donos N, Kostopoulos L, Karring T (2002). Alveolar ridge augmentation using a resorbable copolymer membrane and autogenous bone grafts. Clinical Oral Implants Research 13(2):203-213.

Donos N, Kostopoulos L, Tonetti M, Karring T, Lang NP (2006). The effect of enamel matrix proteins and deproteinized bovine bone mineral on heterotopic bone formation. Clinical Oral Implants Research 17(4):434-438.

Donos N, Lang NP, Karoussis IK, Bosshardt D, Tonetti M, Kostopoulos L (2004). Effect of GBR in combination with deproteinized bovine bone mineral and/or enamel matrix proteins on the healing of critical size defects. Clinical Oral Implants Research 15(1):101-111.

Donos N, Mardas N, Chadha V (2008). Clinical outcomes of implants following lateral bone augmentation: systematic assessment of available options (barrier membranes, bone grafts, split osteotomy). Journal of Clinical Periodontology 35(173-202.

Donos N, Retzepi M, Wall I, Hamlet S, Ivanovski S (2011). In vivo gene expression profile of guided bone regeneration associated with a microrough titanium surface. Clinical Oral Implants Research 22(4):390-398.

Donos N, Sculean A, Glavind L, Reich E, Karring T (2003). Wound healing of degree III furcation involvements following guided tissue regeneration and/or EmdogainÆ. Journal of clinical periodontology 30(12):1061-1068.

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, et al. (1996). Increased bone formation in osteocalcin-deficient mice. Nature 382(6590):448-452.

Elford PR, Guenther HL, Felix R, Cecchini MG, Fleisch H (1987). Transforming growth factor-[beta] reduces the phenotypic expression of osteoblastic MP3T3-E1 cells in monolayer culture. Bone 8(4):259-262.

Ellegaard B, Lee H (1971). New attachment of periodontal tissues after treatment of intrabony lesions. Journal of periodontology 42(10):648-952.

Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, et al. (1985). Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. Proceedings of the National Academy of Sciences of the United States of America 82(19):6507-6511.

Feve B (2005). Adipogenesis: cellular and molecular aspects. Best Practice & Research Clinical Endocrinology & Metabolism 19(4):483-499.

Fincham AG, Luo W, Moradian-Oldak J, Paine ML, Snead ML, Zeichner-David M (2000). Enamel biomineralization: the assembly and disassembly of the protein extracellular organic matrix. Development, Function and Evolution of Teeth:37ñ61.

Fincham AG, Moradian-Oldak J, Sarte PE (1994). Mass-spectrographic analysis of a porcine amelogenin identifies a single phosphorylated locus. Calcified tissue international 55(5):398-400.

Fincham AG, Moradianoldak J (1993). Amelogenin Post-translational Modifications: Carboxy-Terminal Processing and the Phosphorylation of Bovine and Porcine. Biochemical and biophysical research communications 197(1):248-255.

Flamme I, Frolich T, Risau W (1997). Molecular mechanisms of vasculogenesis and embryonic angiogenesis. Journal of cellular physiology 173(2):206-210.

Flemmig TF (1999). Periodontitis: Ann.Periodontol. 4., pp. 32-36.

Frank NY, Schatton T, Kim S, Zhan Q, Wilson BJ, Ma J, et al. (2011). VEGFR-1 Expressed by Malignant Melanoma-Initiating Cells Is Required for Tumor Growth. Cancer Research 71(4):1474.

Forsman, K., L. Lind, Bockman, B., Westermarl, E., Holmgren, G. (1994). Localization of a gene for autosomal dominant amelogenesis imperfecta (ADAI) to chromosome 4q. Human molecular genetics 3(9): 1621.

Frostick SP, Yin Q, Kemp GJ (1998). Schwann cells, neurotrophic factors, and peripheral nerve regeneration. Microsurgery 18(7):397-405.

Fujii S, Maeda H, Wada N, Tomokiyo A, Saito M, Akamine A (2008). Investigating a clonal human periodontal ligament progenitor/stem cell line in vitro and in vivo. Journal of cellular physiology 215(3):743-749.

Fukae M, Tanabe T (1987). Nonamelogenin components of porcine enamel in the protein fraction free from the enamel crystals. Calcified tissue international 40(5):286-293.

Furlanetto RW (1988). Receptor-mediated endocytosis and lysosomal processing of insulin-like growth factor I by mitogenically responsive cells. Endocrinology 122(5):2044-2053.

Gang EJ, Jeong JA, Han S, Yan Q, Jeon CJ, Kim H (2006). In vitro endothelial potential of human UC blood-derived mesenchymal stem cells. Cytotherapy 8(3):215-227.

Garrett S (1996). Periodontal regeneration around natural teeth. Annals of periodontology/the American Academy of Periodontology 1(1):621-666.

Geiger M, Li RH, Friess W (2003). Collagen sponges for bone regeneration with rhBMP-2. Advanced drug delivery reviews 55(12):1613-1629.

Gestrelius S, Andersson C, Johansson AC, Persson E, Brodin A, Rydhag L, et al. (1997). Formulation of enamel matrix derivative for surface coating. Journal of Clinical Periodontology 24(9):678-684.

Gestrelius S, Andersson C, Lidstrom D, Hammarstrom L, Somerman M (1997). In vitro studies on periodontal ligament cells and enamel matrix derivative. Journal of Clinical Periodontology 24(9):685-692.

Giannobile WV, Hernandez RA, Finkelman RD, Ryarr S, Kiritsy CP, D'Andrea M, et al. (2006). Comparative effects of plateletderived growth factor-BB and insulin-like growth factor-I, individually and in combination, on periodontal regeneration in Macaca fascicularis. Journal of periodontal research 31(5):301-312.

Gkranias ND, Graziani F, Sculean A, Donos N (2010). Wound healing following regenerative procedures in furcation degree III defects: histomorphometric outcomes. Clinical Oral Investigations:1-11.

Goldring MB, Tsuchimochi K, Ijiri K (2006). The control of chondrogenesis. Journal of Cellular Biochemistry 97(1):33-44.

Goldstein LJ, Brown SM (1977). The low-density lipoprotein pathway and its relation to atherosclerosis. Annual review of biochemistry 46(1):897-930.

Greenberg S (1995). Signal transduction of phagocytosis. Trends in cell biology 5(3):93-99.

Griffiths GS, Duffy S, Eaton KA, Gilthorpe MS, Johnson NW (2001). Prevalence and extent of lifetime cumulative attachment loss (LCAL) at different thresholds and associations with clinical variables: changes in a population of young male military recruits over 3 years. Journal of Clinical Periodontology 28(10):961-969.

Grinnell F (1984). Fibronectin and wound healing. Journal of cellular biochemistry 26(2):107-116.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. (2002). Stem cell properties of human dental pulp stem cells. Journal of Dental Research 81(8):531-535.

Gronthos S, Zannettino ACW, Hay SJ, Shi S, Graves SE, Kortesidis A, et al. (2003). Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. Journal of Cell Science 116(1827-1835.

Groves E, Dart AE, Covarelli V, Caron E (2008). Molecular mechanisms of phagocytic uptake in mammalian cells. Cellular and Molecular Life Sciences 65(13):1957-1976.

Gruenbaum Cohen Y, Tucker AS, Haze A, Shilo D, Taylor AL, Shay B, et al. (2009). Amelogenin in cranio facial development: the tooth as a model to study the role of amelogenin during embryogenesis. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312(5):445-457.

Guida L, Annunziata M, Carinci F, Di Feo A, Passaro I, Oliva A (2007). In vitro biologic response of human bone marrow stromal cells to enamel matrix derivative. Journal of periodontology 78(11):2190-2196.

Hagenaars S, Louwerse PHG, Timmerman MF, Van der Velden U, Van der Weijden GA (2004). Soft tissue wound healing following periodontal surgery and EmdogainÆ application. Journal of Clinical Periodontology 31(10):850-856.

Hakki SS, Berry JE, Somerman MJ (2001). The effect of enamel matrix protein derivative on follicle cells in vitro. Journal of periodontology 72(5):679-687.

Hakkinen L, Larjava H (1992). Characterization of fibroblast clones from periodontal granulation tissue in vitro. Journal of dental research 71(12):1901.

Hall SE, Savill JS, Henson PM, Haslett C (1994). Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. The Journal of Immunology 153(7):3218.

Hama H, Azuma H, Seto H, Kido J, Nagata T (2008). Inhibitory effect of enamel matrix derivative on osteoblastic differentiation of rat calvaria cells in culture. Journal of periodontal research 43(2):179-185.

Hammarstrom L (1997a). Enamel matrix, cementum development and regeneration. Journal of Clinical Periodontology 24(9):658-668.

Hammarstrom L, Heijl L, Gestrelius S (1997b). Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. Journal of Clinical Periodontology 24(9):669-677.

Hammerle CHF, Schmid J, Lang NP, Olah AJ (1995). Temporal dynamics of healing in rabbit cranial defects using guided bone regeneration. Journal of oral and maxillofacial surgery 53(2):167-174.

Hanahan D (1997). Signaling vascular morphogenesis and maintenance. Science-AAAS-Weekly Paper Edition 277(5322):48-49.

Harada F, Hoshino N, Hanada K, Kawano Y, Atsumi Y, Wakisaka S, et al. (2003). The involvement of brain-derived neurotrophic factor (BDNF) in the regeneration of periodontal Ruffini endings following transection of the inferior alveolar nerve. Archives of histology and cytology 66(2):183-194.

Hassell TM (1993). Tissues and cells of the periodontium. Periodontology 2000 3(9-38.

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI (1992). Characterization of cells with osteogenic potential from human marrow. Bone 13(1):81-88.

Haze, A., A. L. Taylor, et al. (2009). Regeneration of bone and periodontal ligament induced by recombinant amelogenin after periodontitis. Journal of cellular and molecular medicine 13(6): 1110-1124.

Heiji L (1997). Periodontal regeneration with enamel matrix derivative in one human experimental defect. Journal of Clinical Periodontology 24(9):693-696.

Heijl L, Heden G, (1997). Enamel matrix derivative (Emdogain) in the treatment of intrabony periodontal defects. Journal of clinical periodontology 24(9):705-714.

Heng NHM, N'Guessan PD, Kleber BM, Bernimoulin JP, Pischon N (2007). Enamel matrix derivative induces connective tissue growth factor expression in human osteoblastic cells. Journal of periodontology 78(12):2369-2379.

Hentze H, Soong PL, Wang ST, Phillips BW, Putti TC, Dunn N (2009). Teratoma formation by human embryonic stem cells: Evaluation of essential parameters for future safety studies. Stem Cell Research 2(3):198-210.

Heritier M (1982). Experimental induction of cementogenesis on the enamel of transplanted mouse tooth germs. Archives of Oral Biology 27(2):87-97.

Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO, et al. (2004). Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. Journal of cell science 117(19):4411-4422.

Heydarkhan-Hagvall S, Schenke-Layland K, Yang JQ, Heydarkhan S, Xu Y, Zuk PA, et al. (2008). Human adipose stem cells: a potential cell source for cardiovascular tissue engineering. Cells Tissues Organs 187(4):263-274.

Hipp J, Atala A (2008). Sources of stem cells for regenerative medicine. Stem Cell Reviews and Reports 4(1):3-11.

Hiroshima K, Maeda T, Hanada K, Wakisaka S (1998). Calretinin-like immunoreactivity in the regenerating periodontal Ruffini endings of the rat incisor following injury to the inferior alveolar nerve. Brain research 807(1-2):218-221.

Hu CC, Fukae M, Uchida T, Qian Q, Zhang CH, Ryu OH, et al. (1997). Cloning and characterization of porcine enamelin mRNAs. Journal of Dental Research 76(11):1720-1729.

Hu CC, Fukae M, Uchida T, Qian Q, Zhang CH, Ryu OH, et al. (1997). Sheathlin: cloning, cDNA/polypeptide sequences, and immunolocalization of porcine enamel sheath proteins. Journal of Dental Research 76(2):648-657.

Huang TF, Chen YT, Yang TH, Chen LL, Chiou SH, Tsai TH, et al. (2008). Isolation and characterization of mesenchymal stromal cells from human anterior cruciate ligament. Cytotherapy 10(8):806-814.

Huang TF, Chen YT, Yang TH, Chen LL, Chiou SH, Tsai TH, et al. (2008). Isolation and characterization of mesenchymal stromal cells from human anterior cruciate ligament. Cytotherapy 10(8):806-814.

Husmann I, Soulet L, Gautron J, Martelly I, Barritault D (1996). Growth factors in skeletal muscle regeneration. Cytokine & growth factor reviews 7(3):249-258.

Igarashi Y, Aita M, Suzuki A, Nandasena T, Kawano Y, Nozawa-Inoue K, et al. (2007). Involvement of GDNF and its receptors in the maturation of the periodontal Ruffini endings. Neuroscience letters 412(3):222-226.

Imai T, Atsumi Y, Matsumoto K, Yura Y, Wakisaka S (2003). Regeneration of periodontal Ruffini endings of rat lower incisors following nerve cross-anastomosis with mental nerve. Brain research 992(1):20-29.

Irwin CR, Schor SL, Ferguson MWJ (1994). Effects of cytokines on gingival fibroblasts in vitro are modulated by the extracellular matrix. Journal of periodontal research 29(5):309-317.

Iwata T, Morotome Y, Tanabe T, Fukae M, Ishikawa I, Oida S (2002). Noggin blocks osteoinductive activity of porcine enamel extracts. Journal of Dental Research 81(6):387-391.

Jabbar S, Harada F, Aita M, Ohishi M, Saito I, Kawano Y, et al. (2007). Involvement of neurotrophin 4/5 in regeneration of the periodontal Ruffini endings at the early stage. The Journal of Comparative Neurology 501(3):400-412.

Jaffe EA, Nachman RL, Becker CG, Minick CR (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. Journal of Clinical Investigation 52(11):2745-2756.

Jiang J, Safavi KE, Spangberg LSW, Zhu Q (2001). Enamel matrix derivative prolongs primary osteoblast growth. Journal of Endodontics 27(2):110-112.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41-49.

Johansson H, Sjalander P, Sojka P (1991). A sensory role for the cruciate ligaments. Clinical orthopaedics and related research 268):161.

Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes & development 10(24):3129-3140.

Johnson DL, Carnes D, Steffensen B, Cochran DL (2009). Cellular effects of enamel matrix derivative are associated with different molecular weight fractions following separation by size-exclusion chromatography. Journal of periodontology 80(4):648-656.

Johnson GL, Vaillancourt RR (1994). Sequential protein kinase reactions controlling cell growth and differentiation. Current opinion in cell biology 6(2):230-238.

Jones N, Iljin K, Dumont DJ, Alitalo K (2001). Tie receptors: new modulators of angiogenic and lymphangiogenic responses. Nature Reviews Molecular Cell Biology 2(4):257-267.

Jortikka L, Laitinen M, Lindholm TS, Marttinen A (1997). Internalization and intracellular processing of bone morphogenetic protein (BMP) in rat skeletal muscle myoblasts (L6). Cellular signalling 9(1):47-51.

Kamma JJ, Baehni PC (2003). Five-year maintenance follow-up of early-onset periodontitis patients. Journal of Clinical Periodontology 30(6):562-572.

Kanazashi M, Gomi K, Nagano T, Tanabe T, Arai T, Fukae M (2006). The 17-kDa sheath protein in enamel proteins induces cementum regeneration in experimental cavities created in a buccal dehiscence model of dogs. Journal of periodontal research 41(3):193-199.

Kannari K, Sato O, Maeda T, Iwanaga T, Fujita T (1991). A possible mechanism of mechanoreception in Ruffini endings in the periodontal ligament of hamster incisors. The Journal of Comparative Neurology 313(2):368-376.

Karring T, Nyman S, Gottlow J, Laurell L (1993). Development of the biological concept of guided tissue regeneration--animal and human studies. Periodontology 2000 1(1):26-35.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, et al. (1994). Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. The Journal of cell biology 127(6):1755-1766.

Kauvar AS, Thoma DS, Carnes DL, Cochran DL (2010). In Vivo Angiogenic Activity of Enamel Matrix Derivative. Journal of periodontology 81(8):1196-1201.

Kawana F, Sawae Y, Sahara T, Tanaka S, Debari K, Shimizu M, et al. (2001). Porcine enamel matrix derivative enhances trabecular bone regeneration during wound healing of injured rat femur. The Anatomical Record 264(4):438-446.

Kawanabe N, Murata S, Murakami K, Ishihara Y, Hayano S, Kurosaka H, et al. (2009). Isolation of multipotent stem cells in human periodontal ligament using stage-specific embryonic antigen-4. Differentiation 76(2):74-83.

Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM (2001). Enamel matrix derivative (EMDOGAIN) rapidly stimulates phosphorylation of the MAP kinase family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. Journal of Periodontal Research 36(6):367-376.

Keila S, Nemcovsky CE, Moses O, Artzi Z, Weinreb M (2004). In vitro effects of enamel matrix proteins on rat bone marrow cells and gingival fibroblasts. Journal of dental research 83(2):134-138.

Kraan MC, Haringman JJ, Post WJ, Versendaal J, Breedveld FC, Tak PP (1999). Immunohistological analysis of synovial tissue for differential diagnosis in early arthritis. Rheumatology 38(11):1074-1080.

Kriegstein AR, Dichter MA (1983). Morphological classification of rat cortical neurons in cell culture. Journal of Neuroscience 3(8):1634-1647.

Kulyk WM, Franklin JL, Hoffman LM (2000). Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Experimental Cell Research 255(2):327-332.

Kuru L, Parkar MH, Griffiths GS, Newman HN, Olsen I (1998). Flow cytometry analysis of gingival and periodontal ligament cells. Journal of dental research 77(4):555-564.

Lamalice L, Le Boeuf F, Huot J (2007). Endothelial cell migration during angiogenesis. Circulation research 100(6):782-794.

Lang N, Bartold PM, Cullinan M, Jeffcoat M, Mombelli A, Murakami S, et al. (1999). Consensus report: aggressive periodontitis. Annals of Periodontology 4(1):53-53.

Larrivee B, Karsan A (2000). Signaling pathways induced by vascular endothelial growth factor (review). International journal of molecular medicine 5(5):447-456.

Laurell L, Bose M, Graziani F, Tonetti M, Berglundh T (2006). The structure of periodontal tissues formed following guided tissue regeneration therapy of intra bony defects in the monkey. Journal of clinical periodontology 33(8):596-603.

Lee MY, Huang JP, Chen YY, Aplin JD, Wu YH, Chen CY, et al. (2009). Angiogenesis in Differentiated Placental Multipotent Mesenchymal Stromal Cells Is Dependent on Integrin 5 1. 4(10):e6913.

Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, et al. (2007). Autocrine VEGF signaling is required for vascular homeostasis. Cell 130(4):691-703.

Lee SK, Kim YS, Oh HS, Yang KH, Kim EC, Chi JG (2001). Prenatal development of the human mandible. The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology 263(3):314-325.

Lee W, Sodek J, McCulloch CAG (1996). Role of integrins in regulation of collagen phagocytosis by human fibroblasts. Journal of cellular physiology 168(3):695-704.

Lemoli RM, Bertolini F, Cancedda R, De Luca M, Del Santo A, Ferrari G, et al. (2005). Stem cell plasticity: time for a reappraisal? Haematologica 90(3):360-381.

Lindhe J, Karring T, Lang NP (1997). Clinical periodontology and implant dentistry: Munksgaard.

Lindvall O, Kokaia Z (2006). Stem cells for the treatment of neurological disorders. Nature 441(7097):1094-1096.

Listgarten MA (1972). Electron microscopic study of the junction between surgically denuded root surfaces and regenerated periodontal tissues. Journal of Periodontal Research 7(1):68-90.

Liu B, Hattori N, Jiang B, Nakayama Y, Zhang NY, Wu B, et al. (2004). Single cell RT-PCR demonstrates differential expression of GABAC receptor [rho] subunits in rat hippocampal pyramidal and granule cells. Molecular brain research 123(1-2):1-6.

Loe H, Brown LJ (1991). Early onset of periodontitis in the United State of America. Journal of periodontology 62(10):608-616.

Lossdorfer S, Sun M, Gotz W, Dard M, Jager A (2007). Enamel matrix derivative promotes human periodontal ligament cell differentiation and osteoprotegerin production in vitro. Journal of dental research 86(10):980-985.

Lowell CA (2006). Rewiring phagocytic signal transduction. Immunity 24(3):243-245.

Luthman J, Friskopp J, Dahliˆf G, Ahlstrˆm U, Sjˆstrˆm L, Johansson O (1989). Immunohistochemical study of neurochemical markers in gingiva obtained from periodontitis-affected sites. Journal of Periodontal Research 24(4):267-278.

Lutolf S, Radtke F, Aguet M, Suter U, Taylor V (2002). Notch1 is required for neuronal and glial differentiation in the cerebellum. Development 129(2):373-385.

Lynch SE, De Castilla GR, Williams RC, Kiritsy CP, Howell TH, Reddy MS, et al. (1991). The effects of short-term application of a combination of platelet-derived and insulin-like growth factors on periodontal wound healing. Journal of periodontology 62(7):458-467.

Macchiarini P, Jungebluth P, Go T, Asnaghi M, Rees LE, Cogan TA, et al. (2008). Clinical transplantation of a tissue-engineered airway. The Lancet 372(9655):2023-2030.

Madri JA, Pratt BM, Tucker AM (1988). Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. The Journal of cell biology 106(4):1375-1384.

Maeda T, Ochi K, Nakakura-Ohshima K, Youn SH, Wakisaka S (1999). The Ruffini ending as the primary mechanoreceptor in the periodontal ligament: its morphology, cytochemical features, regeneration, and development. Critical Reviews in Oral Biology & Medicine 10(3):307-327.

Mallard F, Tang BL, Galli T, Tenza D, Saint-Pol A, Yue X, et al. (2002). Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. The Journal of cell biology 156(4):653-664.

Manolagas SC (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocrine reviews 21(2):115-137.

Mardas N, Kostopoulos L, Karring T (2002). Bone and suture regeneration in calvarial defects by e-PTFE-membranes and demineralized bone matrix and the impact on calvarial growth: An experimental study in the rat. Journal of Craniofacial Surgery 13(3):453-462.

Mardas N, Stavropoulos A, Karring T (2008). Calvarial bone regeneration by a combination of natural anorganic bovine derived hydroxyapatite matrix coupled with a

synthetic cell binding peptide (PepGenô): an experimental study in rats. Clinical Oral Implants Research 19(10):1010-1015.

Mareddy S, Crawford R, Brooke G, Xiao Y (2007). Clonal isolation and characterization of bone marrow stromal cells from patients with osteoarthritis. Tissue engineering 13(4):819-829.

Margolis HC, Beniash E, Fowler CE (2006). Role of macromolecular assembly of enamel matrix proteins in enamel formation. Journal of dental research 85(9):775-793.

Maru Y, Yamaguchi S, Shibuya M (1998). Flt-1, a receptor for vascular endothelial growth factor, has transforming and morphogenic potentials. Oncogene 16(20):2585-2595.

Matthews B, Holland GR (1975). Coupling between nerves in teeth. Brain research 98(2):354-358.

Maycock J, Wood SR, Brookes SJ, Shore RC, Robinson C, Kirkham J (2002). Characterization of a Procine Amelogenin Preparation, EMADOGAIN, a Biological Treatment for Periodontal Disease. Connective tissue research 43(2):472-476.

McCulloch CAG, Nemeth E, Lowenberg B, Melcher AH (1987). Paravascular cells in endosteal spaces of alveolar bone contribute to periodontal ligament cell populations. The Anatomical Record 219(3):233-242.

Melcher AH, Bowen WH (1969). Biology of the Periodontium: Not Avail.

Mellonig JT (1999). Enamel matrix derivative for periodontal reconstructive surgery: technique and clinical and histologic case report. The International journal of periodontics & restorative dentistry 19(1):8-19.

Menicanin D, Bartold PM, Zannettino ACW, Gronthos S (2010). Identification of a common gene expression signature associated with immature clonal mesenchymal cell populations derived from bone marrow and dental tissues. Stem Cells and Development 19(10):1501-1510.

Messenger MP, Raif EM, Seedhom BB, Brookes SJ (2007). The potential use of enamel matrix derivative for in situ anterior cruciate ligament tissue engineering: a translational in vitro investigation. Tissue Engineering 13(8):2041-2051.

Miyazono K (2000). Positive and negative regulation of TGF-beta signaling. Journal of cell science 113(1101-1109.

Molloy T, Wang Y, Murrell GAC (2003). The roles of growth factors in tendon and ligament healing. Sports medicine 33(5):381-394.

Moradian-Oldak J, Simmer JP, Sarte PE, Zeichner-David M, Fincham AG (1994). Specific cleavage of a recombinant murine amelogenin at the carboxy-terminal region by a proteinase fraction isolated from developing bovine tooth enamel. Archives of Oral Biology 39(8):647-656.

Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, et al. (2006). Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127(6):1151-1165.

Mukherjee S, Ghosh RN, Maxfield FR (1997). Endocytosis. Physiological Reviews 77(3):759-803.

Muller AM, Hermanns M, Skrzynski C, Nesslinger M, M¸ller KM, Kirkpatrick CJ (2002). Expression of the endothelial markers PECAM-1, vWf, and CD34 in vivo and in vitro. Experimental and molecular pathology 72(3):221-229.

Mumulidu A, Hildebrand B, Fabi B, Hammarstrom L, Cochran DL, Dard M, et al. (2007). Purification and analysis of a 5 kDa component of enamel matrix derivative. Journal of Chromatography B 857(2):210-218.

Murphy DJ, Vance J (1999). Mechanisms of lipid-body formation. Trends in biochemical sciences 24(3):109-115.

Murphy KG, Gunsolley JC (2003). Guided tissue regeneration for the treatment of periodontal intrabony and furcation defects. A systematic review. Annals of Periodontology 8(1):266-302.

Nagano T, Iwata T, Ogata Y, Tanabe T, Gomi K, Fukae M, et al. (2004). Effect of heat treatment on bioactivities of enamel matrix derivatives in human periodontal ligament (HPDL) cells. Journal of Periodontal Research 39(4):249-256.

Nagano T, Oida S, Suzuki S, Iwata T, Yamakoshi Y, Ogata Y, et al. (2006). Porcine enamel protein fractions contain transforming growth factor- 1. Journal of periodontology 77(10):1688-1694.

Nagatomo K, Komaki M, Sekiya I, Sakaguchi Y, Noguchi K, Oda S, et al. (2006). Stem cell properties of human periodontal ligament cells. Journal of periodontal research 41(4):303-310.

Nandasena BGTL, Suzuki A, Aita M, Kawano Y, Nozawa-Inoue K, Maeda T (2007). Immunolocalization of aquaporin-1 in the mechanoreceptive Ruffini endings in the periodontal ligament. Brain research 1157(32-40.

Narukawa M, Suzuki N, Takayama T, Shoji T, Otsuka K, Ito K (2006). Enamel matrix derivative stimulates chondrogenic differentiation of ATDC5 cells. Journal of periodontal research 42(2):131-137.

Narukawa M, Suzuki N, Takayama T, Yamashita Y, Otsuka K, Ito K (2007). Enamel matrix derivative stimulates osteogenesis-and chondrogenesis-related transcription factors in C3H10T1/2 cells. Acta Biochimica et Biophysica Sinica 39(1):1-7.

Nave KA, Trapp BD (2008). Axon-glial signaling and the glial support of axon function. Annu. Rev. Neurosci. 31(535-561.

Needleman I, Tucker R, Giedrys-Leeper E, Worthington H (2005). Guided tissue regeneration for periodontal intrabony defectsña Cochrane Systematic Review*. Periodontology 2000 37(1):106-123.

New DAT (1955). A new technique for the cultivation of the chick embryo in vitro. Journal of Embryology and Experimental Morphology 3(4):326-331.

Ng CT, Biniecka M, Kennedy A, McCormick J, FitzGerald O, Bresnihan B, et al. (2010). Synovial tissue hypoxia and inflammation in vivo. Annals of the rheumatic diseases 69(7):1389-1395.

Niemann C, Watt FM (2002). Designer skin: lineage commitment in postnatal epidermis. Trends in cell biology 12(4):185-192.

Ning H, Liu G, Lin G, Garcia M, Li LC, Lue TF, et al. (2009). Identification of an aberrant cell line among human adipose tissue-derived stem cell isolates. Differentiation; research in biological diversity 77(2):172-180.

Nishihara T, Koseki T (2004). Microbial etiology of periodontitis. Periodontology 2000 36(1):14-26.

Nishikawa S (2006). Systemic labeling and visualization of dental sensory nerves by the novel fluorescent marker AM1 43. Anatomical Science International 81(3):181-186.

Niwa H, Burdon T, Chambers I, Smith A (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & development 12(13):2048-2060.

Nokhbehsaim M, Winter J, Rath B, J‰ger A, Jepsen S, Deschner J (2011). Effects of enamel matrix derivative on periodontal wound healing in an inflammatory environment in vitro. Journal of Clinical Periodontology 38(5):479-90.

Nomi M, Atala A, Coppi PD, Soker S (2002). Principals of neovascularization for tissue engineering. Molecular aspects of medicine 23(6):463-483.

Ntambi JM, Young-Cheul K (2000). Adipocyte differentiation and gene expression. Journal of Nutrition 130(12):3122S.

Nyman S, Lindhe J, Karring T (1983). Textbook of clinical periodontology. Copenhagen: Munksgaard.

O'Brien J, Wilson I, Orton T, Pognan F (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry 267(17):5421-5426.

Obeso J, Weber J, Auerbach R (1990). A hemangioendothelioma-derived cell line: its use as a model for the study of endothelial cell biology. Laboratory investigation; a journal of technical methods and pathology 63(2):259-269.

Ohyama M, Suzuki N, Yamaguchi Y, Maeno M, Otsuka K, Ito K (2002). Effect of enamel matrix derivative on the differentiation of C2C12 cells. Journal of periodontology 73(5):543-550.

Oikawa T, Hirotani K, Nakamura O, Shudo K, Hiragun A, Iwaguchi T (1989). A highly potent antiangiogenic activity of retinoids. Cancer letters 48(2):157-162.

P. De Miguel M, Fuentes-Juli·n S, Alcaina Y (2010). Pluripotent Stem Cells: Origin, Maintenance and Induction. Stem Cell Reviews and Reports 6):1-17.

Paine CT, Paine ML, Luo W, Okamoto CT, Lyngstadaas SP, Snead ML (2000). A tuftelin-interacting protein (TIP39) localizes to the apical secretory pole of mouse ameloblasts. Journal of Biological Chemistry 275(29):22284-22292.

Palmer RM, Lumsden AGS (1987). Development of periodontal ligament and alveolar bone in homografted recombinations of enamel organs and papillary, pulpal and follicular mesenchyme in the mouse. Archives of Oral Biology 32(4):281-289.

Pandya NM, Dhalla NS, Santani DD (2006). Angiogenesis--a new target for future therapy. Vascular pharmacology 44(5):265-274.

Pankov R, Yamada KM (2002). Fibronectin at a glance. Journal of Cell Science 115(20):3861-3863.

Parodi R, Liuzzo G, Patrucco P, Brunel G, Santarelli GA, Birardi V, et al. (2000). Use of Emdogain in the treatment of deep intrabony defects: 12-month clinical results. Histologic and radiographic evaluation. The International journal of periodontics & restorative dentistry 20(6):584-595.

Pickl WF, Majdic O, Fischer GF, Petzelbauer P, Fae I, Waclavicek M, et al. (1997). MUC18/MCAM (CD146), an activation antigen of human T lymphocytes. The Journal of Immunology 158(5):2107-2115.

Pihlstrom BL, Michalowicz BS, Johnson NW (2005). Periodontal diseases. The Lancet 366(9499):1809-1820.

Pontoriero R, Lindhe J (1995). Guided tissue regeneration in the treatment of degree III furcation defects in maxillary molars. Journal of clinical periodontology 22(10):810-812.

Pontoriero R, Nyman S, Ericsson I, Lindhe J (1992). Guided tissue regeneration in surgically produced furcation defects. Journal of clinical periodontology 19(3):159-163.

Port F BK (2010). Wnt trafficking: new insights into Wnt maturation, secretion and spreading.: Traffic, pp. 1265-1271.

Potten CS (1998). Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philosophical Transactions of the Royal Society B: Biological Sciences 353(1370):821-830.

Preiss-Landl K, Zimmermann R, H‰mmerle G, Zechner R (2002). Lipoprotein lipase: the regulation of tissue specific expression and its role in lipid and energy metabolism. Current opinion in lipidology 13(5):471-481.

Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Ratajczak J, Wojakowski W, et al. (2008). Hunt for pluripotent stem cell-Regenerative medicine search for almighty cell. Journal of autoimmunity 30(3):151-162.

Rathe F, Junker R, Chesnutt BM, Jansen JA (2009). The effect of enamel matrix derivative (EmdogainÆ) on bone formation: a systematic review. Tissue Engineering Part B: Reviews 15(3):215.

Relaix F MC (2009). Muscle stem cells. In: COC Biol. editor, pp. Dec;21(6):748-53. Epub 2009 Nov 20.

Reseland JE, Reppe S, Larsen AM, Berner HS, Reinholt FP, Gautvik KM, et al. (2006). The effect of enamel matrix derivative on gene expression in osteoblasts. European Journal of Oral Sciences 114(205-211.

Retzepi M (2007). Effect of diabetes mellitus on healing following Guided Bone Regeneration.

Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L, Presta M (1995). Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. Developmental biology 170(1):39-49.

Rider C, Mulloy B (2010). Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists. Biochem. J 429(1-12.

Ripamonti U (2007). Recapitulating development: a template for periodontal tissue engineering. Tissue engineering 13(1):51-71.

Ripamonti U, Crooks J, Petit JC, Rueger DC (2001). Periodontal tissue regeneration by combined applications of recombinant human osteogenic protein-1 and bone morphogenetic protein-2. A pilot study in Chacma baboons (Papio ursinus). European Journal of Oral Sciences 109(4):241-248.

Rodrigues TLS, Marchesan JT, Coletta RD, Novaes Jr AB, Grisi MFM, Souza SLS, et al. (2007). Effects of enamel matrix derivative and transforming growth factor- 1 on human periodontal ligament fibroblasts. Journal of clinical periodontology 34(6):514-522.

Rosling B, Nyman S, Lindhe J, Jern B (1976). The healing potential of the periodontal tissues following different techniques of periodontal surgery in plaque-free dentitions. Journal of Clinical Periodontology 3(4):233-250.

Rossi CA, Pozzobon M, Ditadi A, Archacka K, Gastaldello A, Sanna M, et al. (2010). Clonal characterization of rat muscle satellite cells: proliferation, metabolism and differentiation define an intrinsic heterogeneity. PloS one 5(1):e8523.

Ryoo HM, Lee MH, Kim YJ (2006). Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. Gene 366(1):51-57.

Ryu OH, Fincham AG, Hu CC, Zhang C, Qian Q, Bartlett JD, et al. (1999). Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. Journal of Dental Research 78(3):743-750.

Saito K, Konishi I, Nishiguchi M, Hoshino T, Fujiwara T (2008). Amelogenin binds to both heparan sulfate and bone morphogenetic protein 2 and pharmacologically suppresses the effect of noggin. Bone 43(2):371-376.

Sato O, Maeda T, Kobayashi S, Iwanaga T, Fujita T, Takahashi Y (1988). Innervation of periodontal ligament and dental pulp in the rat incisor: an immunohistochemical investigation of neurofilament protein and glia-specific S-100 protein. Cell and tissue research 251(1):13-21.

Saxby MS (1987). Juvenile periodontitis: an epidemiological study in the west Midlands of the United Kingdom. Journal of Clinical Periodontology 14(10):594-598.

Schlueter SR, Carnes Jr DL, Cochran DL (2007). In vitro effects of enamel matrix derivative on microvascular cells. Journal of periodontology 78(1):141-151.

Schmitz JP, Hollinger JO (1986). The critical size defect as an experimental model for craniomandibulofacial nonunions. Clinical orthopaedics and related research 205(299.

Schor AM, Canfield AE, Sutton AB, Arciniegas E, Allen TD (1995). Pericyte differentiation. Clinical orthopaedics and related research 313(81.

Schwartz JD, Rowinsky EK, Youssoufian H, Pytowski B, Wu Y (2010). Vascular endothelial growth factor receptor 1 in human cancer. Cancer 116(S4):1027-1032.

Schwartz Z, Carnes Jr DL, Pulliam R, Lohmann CH, Sylvia VL, Liu Y, et al. (2000). Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. Journal of periodontology 71(8):1287-1296.

Sculean A, Chiantella GC, Windisch P, Donos N (2000). Clinical and histologic evaluation of human intrabony defects treated with an enamel matrix protein derivative (Emdogain). The International journal of periodontics & restorative dentistry 20(4):374-381.

Sculean A, Donos N, Blaes A, Lauermann M, Reich E, Brecx M (1999). Comparison of enamel matrix proteins and bioabsorbable membranes in the treatment of intrabony periodontal defects. A split-mouth study. Journal of periodontology 70(3):255-262.

Sculean A, Donos N, Schwarz F, Becker J, Brecx M, Arweiler NB (2004). Five year results following treatment of intrabony defects with enamel matrix proteins and guided tissue regeneration. Journal of clinical periodontology 31(7):545-549.

Sculean A, Donos N, Windisch P, Brecx M, Gera I, Reich E, et al. (1999). Healing of human intrabony defects following treatment with enamel matrix proteins or guided tissue regeneration. Journal of Periodontal Research 34(6):310-322.

Sculean A, Donos N, Windisch P, Brecx M, Gera I, Reich E, et al. (2006). Healing of human intrabony defects following treatment with enamel matrix proteins or guided tissue regeneration. Journal of periodontal research 34(6):310-322.

Sculean A, Kiss A, Miliauskaite A, Schwarz F, Arweiler NB, Hannig M (2008). Ten-year results following treatment of intra-bony defects with enamel matrix proteins and guided tissue regeneration. Journal of clinical periodontology 35(9):817-824.

Sculean A, Reich E, Chiantella GC, Brecx M (1999). Treatment of intrabony periodontal defects with an enamel matrix protein derivative (Emdogain): a report of 32 cases. The International journal of periodontics & restorative dentistry 19(2):157-163.

Seo BM, Miura M, Gronthos S, Mark Bartold P, Batouli S, Brahim J, et al. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. The Lancet 364(9429):149-155.

Seto ES, Bellen HJ, Lloyd TE (2002). When cell biology meets development: endocytic regulation of signaling pathways. Genes & development 16(11):1314-1336.

Shirai K, Ishisaki A, Kaku T, Tamura M, Furuichi Y (2009). Multipotency of clonal cells derived from swine periodontal ligament and differential regulation by fibroblast growth factor and bone morphogenetic protein. Journal of periodontal research 44(2):238-247.

Simmer JP, Hu CC, Lau EC, Sarte P, Slavkin HC, Fincham AG (1994). Alternative splicing of the mouse amelogenin primary RNA transcript. Calcified tissue international 55(4):302-310.

Simmons PJ, Torok-Storb B (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 78(1):55-62.

Sinanan A, Hunt NP, Lewis MP (2004). Human adult craniofacial muscle-derived cells: neural-cell-adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. Biotechnology and applied biochemistry 40(25-34.

Singhatanadgit W, Donos N, Olsen I (2009). Isolation and characterization of stem cell clones from adult human ligament. Tissue Engineering Part A 15(9):2625-2636.

Singhatanadgit W, Mordan N, Salih V, Olsen I (2008). Changes in bone morphogenetic protein receptor-IB localisation regulate osteogenic responses of human bone cells to bone morphogenetic protein-2. The International Journal of Biochemistry & Cell Biology 40(12):2854-2864.

Singhatanadgit W, Salih V, Olsen I (2006). Up regulation of bone morphogenetic protein receptor IB by growth factors enhances BMP 2 induced human bone cell functions. Journal of cellular physiology 209(3):912-922.

Sirirat M, Kasetsuwan J, Jeffcoat MK (1996). Comparison between 2 surgical techniques for the treatment of early-onset periodontitis. Journal of periodontology 67(6):603-607.

Slavkin HC, Boyde A (1975). Cementum: an epithelial secretory product. J Dent Res 53(157).

Slavkin HC BA (1974). Cementum: an epithelial secretory product?: J Dent Res, pp. 157.

Smith AG (2001). Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol 17(435-62.

Sodek J, McKee MD (2000). Molecular and cellular biology of alveolar bone. Periodontology 2000 24(1):99-126.

Sommerfeldt D, Rubin C (2001). Biology of bone and how it orchestrates the form and function of the skeleton. European Spine Journal 10(86-95.

Staquet MJ, Dezutter-Dambuyant C, Zambruno G, Schmitt D (1989). Human epidermal basal keratinocytes express CDw29 antigens. British Journal of Dermatology 121(5):577-585.

Staton CA, Reed MWR, Brown NJ (2009). A critical analysis of current in vitro and in vivo angiogenesis assays. International Journal of Experimental Pathology 90(3):195-221.

Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Frenkel B, Montecino M (1996). Mechanisms regulating osteoblast proliferation and differentiation. Principles of bone biology:69ñ86.

Stewart R, Nelson J, Wilson DJ (1989). Epidermal growth factor promotes chick embryonic angiogenesis. Cell biology international reports 13(11):957-965.

Suzuki S, Nagano T, Yamakoshi Y, Gomi K, Arai T, Fukae M, et al. (2005). Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-beta. Journal of dental research 84(6):510-514.

Svensson, J., C. Andersson, et al. (2006). Histidine tag fusion increases expression levels of active recombinant amelogenin in Escherichia coli. Protein expression and purification 48(1): 134-141.

Swanson EC, Fong HK, Foster BL, Paine ML, Gibson CW, Snead ML, et al. (2006). Amelogenins regulate expression of genes associated with cementoblasts in vitro. European Journal of Oral Sciences 114(s1):239-243.

Taba Jr M, Jin Q, Sugai JV, Giannobile WV (2005). Current concepts in periodontal bioengineering. Orthodontics & craniofacial research 8(4):292-302.

Tajika Y, Matsuzaki T, Suzuki T, Aoki T, Hagiwara H, Kuwahara M, et al. (2004). Aquaporin-2 is retrieved to the apical storage compartment via early endosomes and phosphatidylinositol 3-kinase-dependent pathway. Endocrinology 145(9):4375-4383.

Takada I, Kouzmenko AP, Kato S (2009). Molecular switching of osteoblastogenesis versus adipogenesis: implications for targeted therapies. Expert Opinion on Therapeutic Targets 13(5):593-603.

Takayama T, Suzuki N, Narukawa M, Tokunaga T, Otsuka K, Ito K (2005). Enamel matrix derivative stimulates core binding factor 1/runt-related transcription factor-2 expression via activation of Smad1 in C2C12 cells. Journal of periodontology 76(2):244-249.

Techawattanawisal W, Nakahama K, Komaki M, Abe M, Takagi Y, Morita I (2007). Isolation of multipotent stem cells from adult rat periodontal ligament by neurosphere-forming culture system. Biochemical and biophysical research communications 357(4):917-923.

Temple S (2001). The development of neural stem cells. Nature 414(6859):112-117.

Ten Cate AR (1996). The role of epithelium in the development, structure and function of the tissues of tooth support. Oral Diseases 2(1):55-62.

Ten Cate AR, Dale AC (1994). Oral histology: development, structure, and function: Mosby St. Louis.

Ten Cate AR, Mills C, Solomon G (1971). The development of the periodontium. A transplantation and autoradiographic study. The Anatomical Record 170(3):365-379.

Tencate AR, Mills C (1972). The development of the periodontium: the origin of alveolar bone. The Anatomical Record 173(1):69-77.

Terranova VP, Goldman HM, Listgarten MA (1990). The periodontal attachment apparatus structure, function, and chemistry. Contemporary periodontics:33.

Thoma DS, Villar CC, Carnes DL, Dard M, Patricia Chun YH, Cochran DL (2011). Angiogenic activity of an enamel matrix derivative (EMD) and EMD derived proteins: an experimental study in mice. Journal of Clinical Periodontology 3(38):253-260.

Thomas ED, Lochte Jr HL, Lu WC, Ferrebee JW (1957). Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. New England Journal of Medicine 257(11):491-496.

Till JE, McCulloch EA (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Research 14(2):213-222.

Tokiyasu Y, Takata T, Saygin E, Somerman M (2000). Enamel factors regulate expression of genes associated with cementoblasts. Journal of periodontology 71(12):1829-1839.

Tomokiyo A, Maeda H, Fujii S, Wada N, Shima K, Akamine A (2007). Development of a multipotent clonal human periodontal ligament cell line. Differentiation 76(4):337-347.

Tonetti MS, Mombelli A (1999). Early-onset periodontitis. Annals of Periodontology 4(1):39-52.

Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J (2011). Functional architecture of Weibel-Palade bodies. Blood. In press.

Vailhe, B., D. Vittet, et al. (2001). In vitro models of vasculogenesis and angiogenesis. Laboratory investigation 81(4): 439-452.

Van der Pauw MT, Van den Bos T, Everts V, Beertsen W (2000). Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor 1 release of periodontal ligament and gingival fibroblasts. Journal of periodontology 71(1):31-43.

Veis A, Tompkins K, Alvares K, Wei K, Wang L, Wang XS, et al. (2000). Specific amelogenin gene splice products have signaling effects on cells in culture and in implants in vivo. Journal of Biological Chemistry 275(52):41263-41272.

Villegas G, Lange-Sperandio B, Tufro A (2005). Autocrine and paracrine functions of vascular endothelial growth factor (VEGF) in renal tubular epithelial cells. Kidney international 67(2):449-457.

Wada Y, Yamamoto H, Nanbu S, Mizuno M, Tamura M (2008). The Suppressive Effect of Enamel Matrix Derivative on Osteocalcin Gene Expression of Osteoblasts Is Neutralized by an Antibody Against TGF. Journal of periodontology 79(2):341-347.

Wakisaka S, Atsumi Y (2003). Regeneration of periodontal Ruffini endings in adults and neonates. Microscopy research and technique 60(5):516-527.

Wang M, Su Y, Sun H, Wang T, Yan G, Ran X, et al. (2010). Induced endothelial differentiation of cells from a murine embryonic mesenchymal cell line C3H/10T1/2 by angiogenic factors in vitro. Differentiation 79(1):21-30.

Wang Y, Pennock SD, Chen X, Kazlauskas A, Wang Z (2004). Platelet-derived growth factor receptor-mediated signal transduction from endosomes. Journal of Biological Chemistry 279(9):8038-8046.

Warotayanont R, Zhu D, Snead ML, Zhou Y (2008). Leucine-rich amelogenin peptide induces osteogenesis in mouse embryonic stem cells. Biochemical and biophysical research communications 367(1):1-6.

Weissman IL (2000). Stem Cells: Units of Development, Review Units of Regeneration, and Units in Evolution. Cell 100(157-168.

Weninger W, Rendl M, Mildner M, Mayer C, Ban J, Geusau A, et al. (2000). Keratinocytes Express the CD146 (Muc18/S-Endo) Antigen in Tissue Culture and During Inflammatory Skin Diseases1. Journal of Investigative Dermatology 115(2):219-224.

Wennstrom JL, Lindhe J (2002). Some effects of enamel matrix proteins on wound healing in the dento gingival region. Journal of Clinical Periodontology 29(1):9-14.

Widera D, Grimm WD, Moebius JM, Mikenberg I, Piechaczek C, Gassmann G, et al. (2007). Highly efficient neural differentiation of human somatic stem cells, isolated by minimally invasive periodontal surgery. Stem Cells and Development 16(3):447-460.

Williams BO, Insogna KL (2009). Where Wnts went: the exploding field of Lrp5 and Lrp6 signaling in bone. Journal of Bone and Mineral Research 24(2):171-178.

Wilting J, Christ B, Weich HA (1992). The effects of growth factors on the day 13 chorioallantoic membrane (CAM): a study of VEGF 165 and PDGF-BB. Anatomy and embryology 186(3):251-257.

Windisch P, Sculean A, Klein F, TÛth V, Gera I, Reich E, et al. (2002). Comparison of Clinical, Radiographic, Histometric Measurements Following Treatment With Guided Tissue Regeneration or Enamel Matrix Proteins in Human Periodontal Defects. Journal of periodontology 73(4):409-417.

Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW, et al. (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature 467(7314):426-429.

Woltering EA, Barrie R, O'Dorisio TM, Arce D, Ure T, Cramer A, et al. (1991). Somatostatin analogues inhibit angiogenesis in the chick chorioallantoic membrane. Journal of Surgical Research 50(3):245-251.

Worch KP, Listgarten MA, Korostoff JM (2001). A multidisciplinary approach to the diagnosis and treatment of early-onset periodontitis: a case report. Journal of periodontology 72(1):96-106.

Wu M, Wei YQ (2004). Development of respiratory stem cells and progenitor cells. Stem Cells and Development 13(6):607-613.

Wu X, Walker J, Zhang J, Ding S, Schultz PG (2004). Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. Chemistry & biology 11(9):1229-1238.

Xu J, Wang W, Kapila Y, Lotz J, Kapila S (2009). Multiple differentiation capacity of STRO-1+/CD146+ PDL mesenchymal progenitor cells. Stem Cells and Development 18(3):487-496.

Ying QL, Nichols J, Chambers I, Smith A (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115(3):281-292.

Yoneda S, Itoh D, Kuroda S, Kondo H, Umezawa A, Ohya K, et al. (2003). The effects of enamel matrix derivative (EMD) on osteoblastic cells in culture and bone regeneration in a rat skull defect. Journal of Periodontal Research 38(3):333-342.

Yoshikawa DK, Kollar EJ (1981). Recombination experiments on the odontogenic roles of mouse dental papilla and dental sac tissues in ocular grafts. Archives of Oral Biology 26(4):303-307.

Yuan Q, Gong P, Tan Z (2007). Schwann cell graft: A method to promote sensory responses of osseointegrated implants. Medical hypotheses 69(4):800-803.

Yue X, Tomanek RJ (2001). Effects of VEGF165 and VEGF121 on vasculogenesis and angiogenesis in cultured embryonic quail hearts. American Journal of Physiology-Heart and Circulatory Physiology 280(5):H2240-H2247.

Yukna RA, Mellonig JT (2000). Histologic evaluation of periodontal healing in humans following regenerative therapy with enamel matrix derivative. A 10-case series. Journal of periodontology 71(5):752-759.

Zhang H, Tompkins K, Garrigues J, Snead ML, Gibson CW, Somerman MJ (2010). Full length amelogenin binds to cell surface LAMP-1 on tooth root/periodontium associated cells. Archives of Oral Biology 55(6):417-425.

Zhou Y, Hutmacher DW, Sae-Lim V, Zhou Z, Woodruff M, Lim TM (2008). Osteogenic and adipogenic induction potential of human periodontal cells. Journal of periodontology 79(3):525-534.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue engineering 7(2):211-228.

Appendices

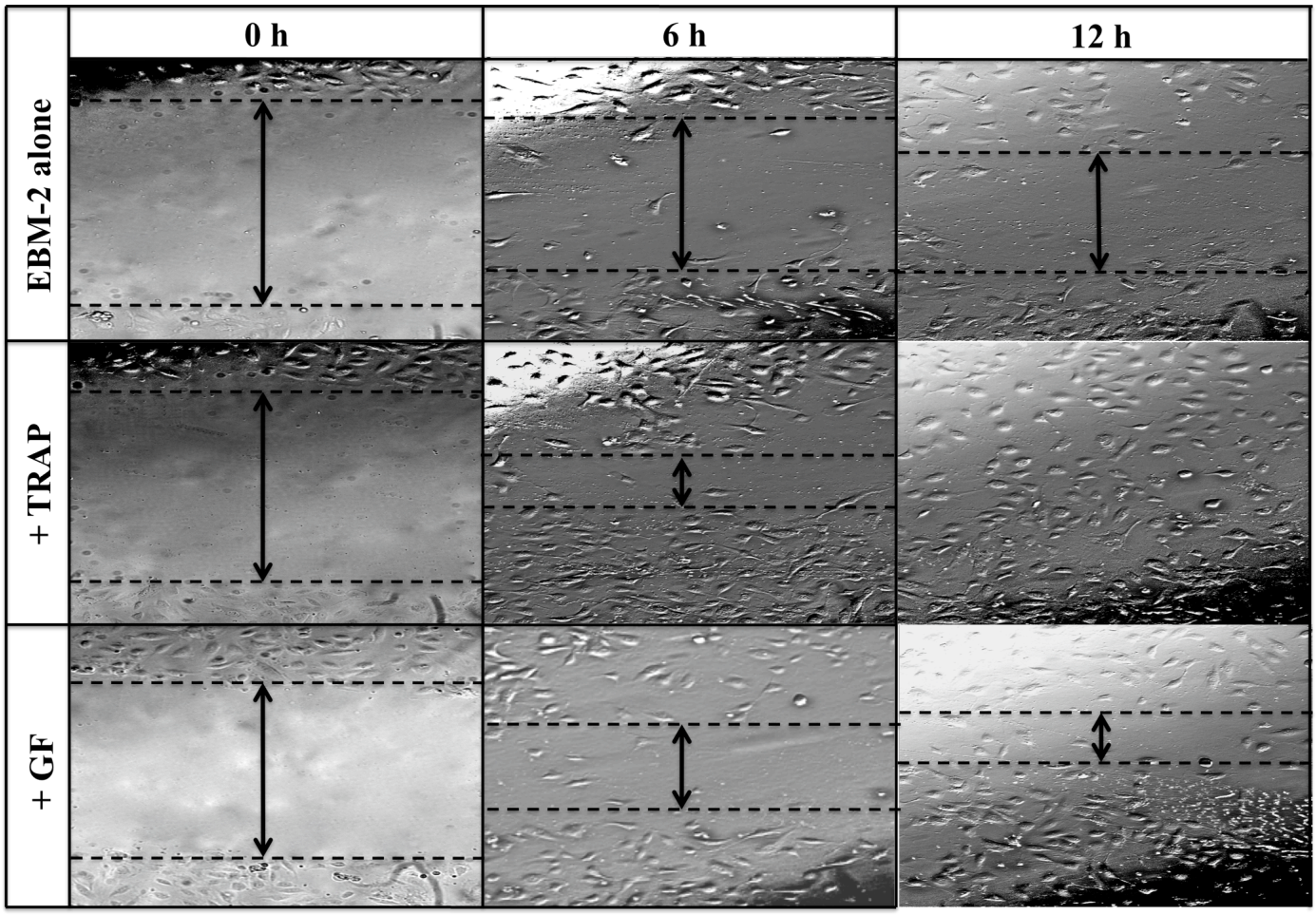
Appendix 1: Supplementary material for Chapter 4

Appendix Material 1.1. Effects of synthetic amelogenin isoforms and peptides on bone-forming cells					
Conc. (µg/ml)	**TRAP**	**LRAP**	**TCT**	**LCT**	**ONT**
0.1	0.9±0.04	1.9±1.40*	0.8±0.06	1.7±1.14*	1.1±0.16
1	0.5±0.09*	4.4±1.17*	0.5±0.07*	5.9±1.88*	1.0±0.22
10	0.6±0.10*	3.9±1.30*	0.7±0.07*	5.5±2.18*	1.2±0.29
100	0.5±0.16*	3.2±1.43*	0.6±0.12*	4.9±0.77*	0.9±0.11

Effects of TRAP, LRAP, TCT, LCT and ONT on terminal osteogenic differentiation of PDL cells. Alizarin red staining of the cells cultured for 3 weeks in OM in the presence of peptides at concentrations 0.1, 1, 10 and 100 µg/ml for the PDL cells. The numbers are the relative staining intensities compared with OM alone, defined as 1.0.

**Indicates statistically significant difference compared with OM alone $p<0.05$.*

Appendix 2: Supplementary material for Chapter 6



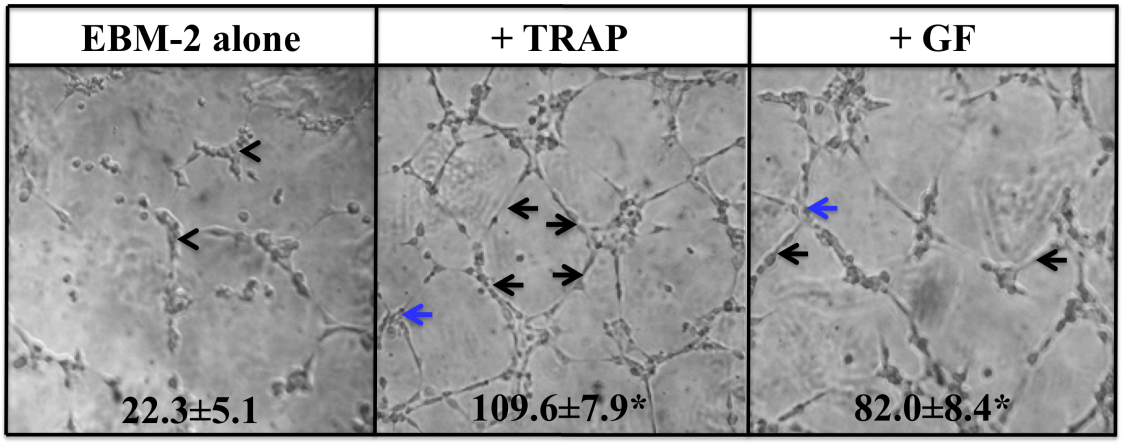
Appendix Material 2.1. Representative microscopic images of HUVEC (positive control cells) cultured in EBM-2 alone, EBM-2 + TRAP (30 µg/ml) and EBM-2 + GF for 6 and 12 h following the creation of an in vitro cell wound, as described in ***Materials and methods****. The area between the black dashed lines shows the size of the wound (space without the cells). Magnification x 10.*

Conditions	Cell migration (in pixels x 10^3)	
	6 h	**12 h**
EBM-2 alone	15.8±3136	21.7±3250
+ TRAP	29.1±3568*	51.2±4275*
+ GF	27.0±5130*	47.8±3204*

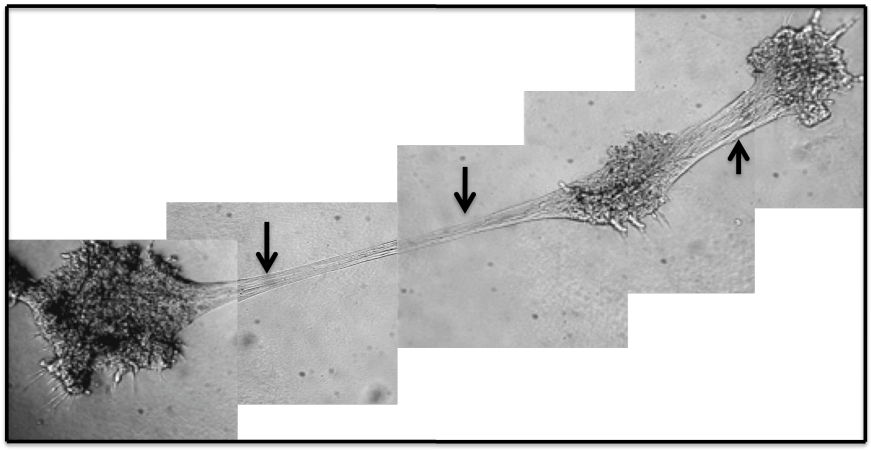
Appendix Material 2.2. Effects of TRAP on migration of HUVEC (positive control cells) when cultured in EBM-2 alone, EBM-2 + TRAP (30 μg/ml) and EBM-2 + GF for 6 and 12 h after creating the cell wound in vitro. The numbers are the average (of 3 separate experiments) pixel counts (x 10^3) of HUVEC migration (pixels of wound (area with no cells) at 0 h minus pixels of wound at 6 or 12 h in the absence and presence of TRAP and GF). Pixels are defined as a point of reference that has a specific recorded location within the micrograph.

**Indicates statistically significant difference compared with EBM-2 alone ($p<0.05$).*

(i)

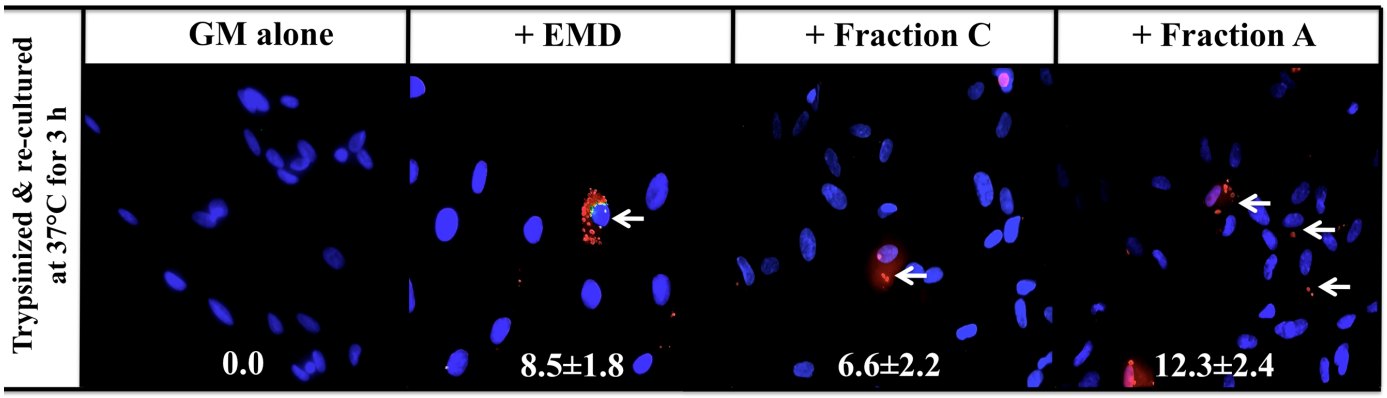


(ii)

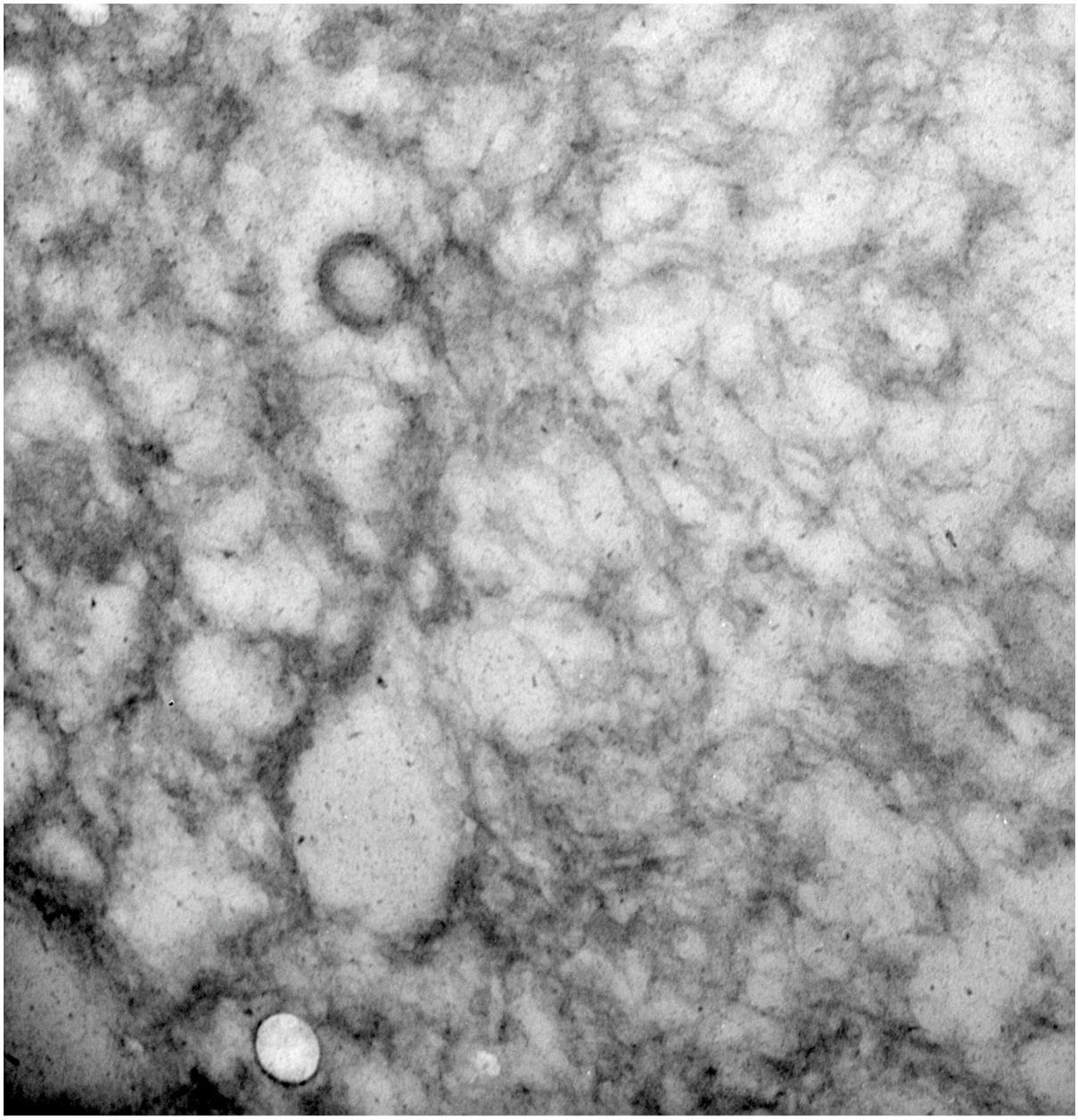


Appendix Material 2.3. Angiogenic structure formation by HUVEC (positive control cells) in vitro. (i) HUVEC cultured for 5 h in EBM-2 alone, EBM-2 + TRAP (30 µg/ml) and EBM-2 + GF. The numbers are the angiogenic structure branching points. The black arrows show the polygonal tubule-like structures formed by HUVEC. The blue arrows show branching points of the HUVEC. Black arrowheads show cells that did not form polygonal tubular-like structures. The values are the mean ±SE of five measurements of three separate experiments. (ii) Elongated BV-like structure formation by EC cultured for 15 h in EBM-2 + TRAP. Magnification x 10.

Appendix 3: Supplementary material for Chapter 8

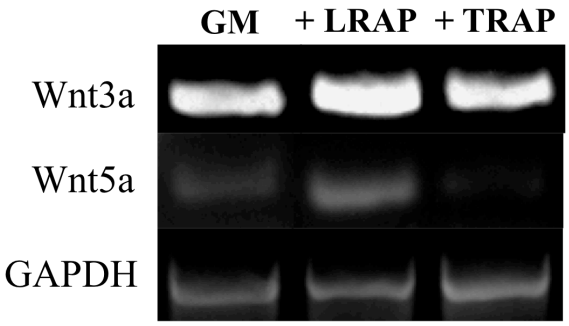


Appendix Material 3.1. Micrographs of the internalization of biotinylated EMD, Fraction C and Fraction A by PDL cells. Cells were incubated at 4°C for 45 min in the absence and presence of EMD, Fraction C and Fraction A, then washed and further incubated at 37°C for 3 h, washed, trypsinized to digest membrane-bound ligands and further cultured for 3 h to allow the cell attachment to the tissue culture plastic and the cells thereafter were reacted with red fluorescent Alexa Fluor-streptavidin. EMD and the EMD Fractions are visualized by red fluorescent staining in PDL cells. The numbers are the % of total cells exhibiting red fluorescent staining. Note the white arrows showing red fluorescent EMD and the EMD Fractions localization within distinct intracellular vesicle-like structures. The nuclei are stained blue using Hoechst dye. Magnification x 20.

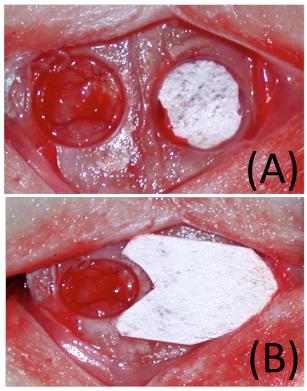


Appendix Material 3.2. Immunogold EM showing the negative control performed LAMP-1. The sample sections were immunogold-stained with non-specific mouse IgG primary antibody and 5 nm gold-goat anti-mouse IgG. Note that no non-specific 5 nm gold particle observed in the negative control section.

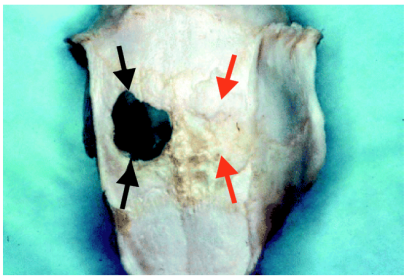
Appendix 4: Supplementary material for Chapter 9



Appendix Material 4.1. Effects of LRAP (1 μg/ml) and TRAP (1 μg/ml) on Wnt3a and Wnt5a genes of PDL cells. Representative RT-PCR gels showing the expression of Wnt3a and 5a by PDL cells cultured for 6 h in GM alone and in the presence of LRAP and TRAP. Note the elevated levels of the Wnt3a and 5a mRNA transcripts of PDL cells cultured in the presence of LRAP. In contrast, note the constant level of Wnt3a and the reduced levels of Wnt5a mRNA transcripts in PDL cells cultured in the presence of TRAP.



Appendix Material 4.2. Demonstration of the creation of standardized, bicortical, critical size (5 mm) calvarial defects at the midportion of each parietal bone in the rat (Donos et al., 2011).



Appendix Material 4.3. Healing of two critical size calvarial defects after 4 months post-operatively. The left defect (untreated control; black arrows) demonstrates little if any bone regeneration occurred. The right defect (red arrows) which was treated with occlusive membranes/GBR exhibited complete bone regeneration (Donos et al., 2004).